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Doctoral Program in Biomedicine (DPBM)

# In Search of Improved Outcome Prediction of Prostate Cancer – A Biological and Clinical Approach

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**ACADEMIC DISSERTATION**

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“… The history of Medicine is replete with examples of cures obtained years, decades, and even centuries before the mechanism of action was understood for these cures.”

- Sidney Farber

# Abbreviations

ACM: Any-Cause Mortality

ADT: Androgen deprivation therapy

AR: Androgen Receptor

AS: Active Surveillance

AUC: Area Under the Curve

BCR: Biochemical Recurrence

Bx: Biopsy

CBx: Confirmatory Biopsy

CCLO: Cumulative number of cancer locations

CRPC: Castration-Resistant Prostate Cancer

DCA: Decision Curve Analysis

Dg: Diagnosis

DgBx: Diagnostic Biopsy

DNA: Deoxyribonucleic acid

DSS: Disease-Specific Survival

EAU: European Association of Urology

EBRT: External Beam Radiotherapy

EHR: Electronic Health Records

ERG: ETS-related gene

ERSPC: European Randomized Study of Screening for Prostate Cancer

FDA: Food and Drug Administration

FU: Follow-up

Fusion Bx: Magnetic Resonance Imaging and Ultrasound Fusion Guided Prostate Biopsy

GG: Grade Group

GS: Gleason Score

GU: Gleason Upgrade

HUS: Helsinki and Uusimaa Hospital District

IEO: European Institute of Oncology

IHC: Immunohistochemistry

IQR: Inner Quartile Range

IRB: Institutional Review Board

LDR: low dose rate

LN: Lymph Node

mCRPC: metastatic Castration-Resistant Prostate Cancer

mpMRI: multiparametric magnetic resonance imaging

MRI: Magnetic-Resonance Imaging

NEPC: Neuroendocrine Prostate Cancer

OS: Overall Survival

PBD: Protocol-Based Discontinuation

PCSM: Prostate Cancer-Specific Mortality

PIN: Personal Identity Number

PIRADS: Prostate Imaging – Reporting and Data System

PLCO: Prostate, Lung, Colorectal, and Ovarian

PRIAS: Prostate cancer Research International: Active Surveillance

PSA: Prostate-specific Antigen

PSA-D: Prostate-specific Antigen Density

PSA-DT: Prostate-Specific Antigen Doubling Time

pT: pathological tumor stage

PTEN: Phosphatase and Tensin homolog

RALP: Robotic-Assisted Laparoscopic Prostatectomy

ROC: Receiver Operating Characteristic

RP: Radical Prostatectomy

RRP: Retropubic Radical Prostatectomy

RT: Radiotherapy

RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction

SSN: Social Security Number

THL: The [Finnish] National Institute for Health and Welfare

TMA: Tissue-Microarray

TRUS: Transrectal ultrasonography

UKM: University Hospital Münster

US: United States

USPSTF: United States Preventive Services Task Force

WHO: World Health Organization

# Original Publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. **Cumulative Cancer Locations is a Novel Metric to Predict Active Surveillance Outcomes: A Multi-Center study**, Erickson A.M.*, Luzzago SL*, Semjonow A., Vasarainen HV, Laajala, TD, Musi G., De Cobelli O, Mirtti T.K., Rannikko A.S. [*Accepted*, European Urology Oncology, April 13th 2018]

II. **PTEN Loss but Not ERG Expression in Diagnostic Biopsies Is Associated with Increased Risk of Progression and Adverse Surgical Findings in Men with Prostate Cancer on Active Surveillance.** Lokman U*, Erickson AM*, Vasarainen H, Rannikko AS, Mirtti T. Eur Urol Focus 2017. doi:10.1016/j.euf.2017.03.004.

III. **Loss of PTEN expression in ERG-negative prostate cancer predicts secondary therapies and leads to shorter disease-specific survival time after radical prostatectomy**, Kanerva Lahdensuo, Andrew Erickson*, Irena Saarinen*, Heikki Seikkula, Johan Lundin, Mikael Lundin, Stig Nordling, Anna Bützow, Hanna Vasarainen, Peter J Boström, Pekka Taimen, Antti Rannikko and Tuomas Mirtti. Modern Pathology. 2016; 29: 1565–1574. http://doi.org/10.1038/modpathol.2016.154 .

**IV.** **New prostate cancer grade grouping system predicts survival after radical prostatectomy**, Andrew Erickson, Kevin Sandeman*, Kanerva Lahdensuo*, Stig Nordling, Markku Kallajoki, Heikki Seikkula, Anna Bützow, Hanna Vasarainen, Peter J. Boström, Pekka Taimen, Antti Rannikko, Tuomas Mirtti. Human Pathology. Human Pathology. 2018; 0. doi:10.1016/j.humpath.2018.01.027.

* These authors contributed equally to this work

# Author's Contributions

The author's own contributions to Studies I-IV are listed as follows.

Study I:

- Concept and design
- Acquisition of Data
- Analysis and interpretation of data
- Drafting of the manuscript
- Critical revision of the manuscript for important intellectual content
- Statistical analysis

Study II:

- Acquisition of Data
- Analysis and interpretation of data
- Drafting of the manuscript
- Statistical analysis

Study III*:

- Acquisition of Data
- Analysis and interpretation of data
- Drafting of the manuscript
- Statistical analysis

Study IV:

- Concept and design
- Acquisition of Data
- Analysis and interpretation of data
- Drafting of the manuscript
- Critical revision of the manuscript for important intellectual content
- Statistical analysis

*Study III has been included in an earlier dissertation (*Diagnostic and Predictive Tools in Localized Prostate Cancer: Biopsies, Magnetic-Resonance Imaging, and Tissue Markers)* and is included here with permission from the first author (permission granted 17.01.2018).

# Abstract

Prostate cancer (PC) is the most common cancer found in western industrialized countries. According to the most recent data, PC accounted for approximately 30% of all new cancer diagnoses of Finnish men. Less than 15% of all diagnosed men, however, will die due to disease. Primary treatments come at the cost of actively-treating a portion of patients who would not die due to disease nor develop any symptoms, even if left untreated. There is an unmet need for better prognostication of patients into more accurate risk-categories. Re-use and novel analysis of existing clinical data, as well as molecular analysis offer a strategy to address this need.

Active surveillance (AS) is an effective treatment option for men with low-risk localized prostate cancer with curative intent. In Study I, We developed a novel metric, cumulative number of cancer locations (CCLO), which is calculated by summing the total positive number of sextant positive locations during the 1-year diagnostic phase of AS. Similarly, current AS inclusion criteria do not take into account molecular profiles of PC at the time of diagnosis. In Study II, we studied whether the ERG and PTEN protein expression, through immunohistochemistry, could better predict poor AS outcomes. In Study III, we studied the same proteins, ERG and PTEN, with regard to stratifying patients by survival profiles in cohorts with long term follow-up, and clinically relevant mortality outcomes. Lastly, In Study IV, we studied the difference between GG (Grade group) and GS (Gleason Score), in large cohorts with long term follow-up and mortality outcomes.

**Main Results: I:** Increased CCLO predicts poor AS outcomes in three independent cohorts. The 3-tier CCLO risk grouping outperforms the current standard of care, the number of positive cores at confirmatory biopsy, in predicting AS outcomes. **II:** PTEN loss in diagnostic biopsy predicts poor AS outcomes. PTEN loss is an independent predictor of poor AS outcomes. **III:** PTEN loss was associated with poor study outcomes. Patients with PTEN loss and ERG negativity were at significantly increased risk for poor outcomes compared to other biomarker status patients. **IV:** GG was independently associated with poor outcomes. In the same study cohort, GG outperformed GS in predicting poor study outcomes.

**Conclusions:** Grade group outperforms Gleason score in predicting prostate cancer patient outcomes and should be adopted into routine

uropathological assessment of prostate cancer. In considering candidates for active surveillance, PTEN immunohistochemical stainings should be performed on diagnostic biopsies, and if PTEN loss is found, treatment change to radical intervention should be considered. If a patient continues on AS to confirmatory systematic biopsy, the number of cancer locations between diagnostic and confirmatory biopsies should be assessed, and if patients are determined to be in the high-risk group of CCLO ≥ 3, treatment change should be considered. Finally, if PTEN loss is found in patient's radical prostatectomy specimens, these patients should be followed more intensively post-operatively. However, further clinical validation is needed, preferably in prospective randomized trials.

# 1. Introduction

Prostate cancer (PC) is one of the most common cancers among men in the western world. In Finland, PC made up 31% of all newly diagnosed cancers in men between 2010-2014 (Engholm *et al*, 2017). However, less than 15% of men diagnosed with PC will die due to disease (Engholm *et al*, 2010; Siegel *et al*, 2017). Many men with PC harbor a relatively harmless form of PC which will never significantly impact the patient but causes them to suffer from harmful side effects of active treatment (Welch & Black, 2010).

Prostate-specific antigen (PSA) is a non--cancer-specific biomarker that has been proposed and studied for use in screening men for PC (Stamey *et al*, 1987; Andriole *et al*, 2009). Results from the Prostate, Lung, Colon, and Ovarian (PLCO) trial in the United States showed no decrease in PC-specific mortality (PCSM) after 10 years of follow-up for men who underwent PSA-screening (Andriole *et al*, 2009), whereas the European Randomized Study of Screening for Prostate Cancer (ERSPC) trial showed a decrease in mortality at 9 years of follow-up (Schröder *et al*, 2009a), and furthermore, a 21% decrease in prostate cancer-specific mortality at 13 years of follow-up (Schröder *et al*, 2014). Thus, while useful in following PC progression after primary therapy, the use of PSA for early detection of PC is controversial. The incidence of PC in the United States has decreased (Siegel *et al*, 2017) after the United States Preventive Services Task Force recommendation (Final Update Summary: Prostate Cancer: Screening - US Preventive Services Task Force, 2016) against PSA-screening. However, recent studies indicate increased presentation of metastatic PC at diagnosis in the United States (Hu *et al*, 2016). As of April 2017, the United States Preventive Services Task Force has new draft recommendations for individualized PSA-screening for men ages 55-69 which will likely again increase detection rates (USPSTF, 2017). In Europe, the recently published ProtecT trial showed there was no difference in outcomes for men with localized diseased randomized to radical prostatectomy, radiotherapy, and active monitoring study outcomes (Hamdy *et al*, 2016). However, approximately three-fourths of the patients were low-risk PC patients by today's standards and, thus, not necessarily considered candidates for immediate radical treatments. Furthermore, approximately one-fourth of the patients were intermediate to high-risk patients not considered candidates for active monitoring by current standards. These findings underscore the need for better prognostic tools in predicting PC outcomes.

Finland offers unique resources to conduct high-impact medical research. Finland, like other Nordic countries Denmark, Sweden, Norway, and Iceland, is a welfare state, with citizens having the universal right to health care (Ministry of Social Affairs and Health, 2013). Quality of treatment is high and allows researchers to study the course of disease progression while controlling for social-determinants of outcome. In the United States, in contrast, social determinants of health are also known risk-factors of disease-severity at diagnosis and outcome. Uninsured patients have been shown to have increased risk of higher Gleason score (Fedewa *et al*, 2010) and distant-metastatic disease (Brawley, 2012) at diagnosis. Despite universal access to care, however, even Finland is not immune to socio-economic disparities in PC outcomes, as a recent publication showed that highly educated men are more likely likely to experience better PC outcomes as compared to less educated men (Seikkula *et al*, 2018).

Despite the increased knowledge of genomics, the clinical translation of genomic data to treatment of PC has been slow. Recent large scale efforts have been focused on advanced PC, such as castration-resistant PC (CRPC) (Grasso *et al*, 2012), neuroendocrine (NE) CRPC (Beltran *et al*, 2016), and metastatic PC (Gundem *et al*, 2015; Pritchard *et al*, 2016; Zehir *et al*, 2017). To our knowledge, one recent major study has been conducted to study genomic alterations in primary PC (Fraser *et al*, 2017) however, this study did not report long term follow-up and outcomes of these patients.

The central aim of this dissertation is to contribute to the ongoing efforts to improve prognostication of PC. Studies I and IV constitute an effort to retrospectively analyze health records and analyze them in novel ways to better predict patient outcomes. Studies II & III focus on studying the molecular profiles of PC, specifically ERG and PTEN, and their ability to predict patient outcomes.

# 2. Literature Review

## 2.1 The Prostate

The prostate is a gland of the male reproductive system. The prostate is located anterior to the rectum, caudally from the bladder (Figure 1A) and surrounds the urethra. The ejaculatory ducts open into the prostatic

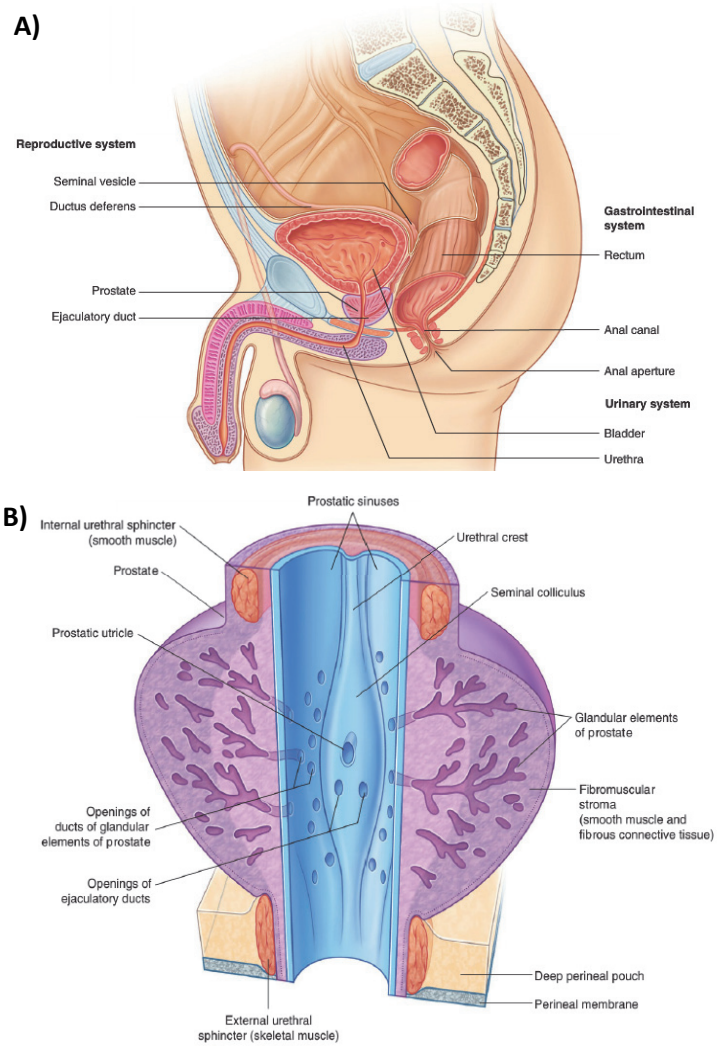


*Figure 1.* Urogenital anatomy (A) and the prostate (B). (Reprinted with permission from Gray's Anatomy for Students 3E, 15022017)

urethra, connecting the reproductive and urinary tracts (Figure 1B) (Drake, R, Vogl, AW, Mitchell, 2015). Prenatally, the prostate develops between the 8th – 36th weeks of gestation, and stays the same size until puberty, when androgens induce growth of the prostate. (Carlson, 2014). The post-pubertal prostate weighs approximately 20g, and remains this size until approximately age 50 (Bostwick & Cheng, 2014).

During ejaculation, sperm-rich fluid secreted from the epididymis mixes with fluids from the seminal vesicles and prostate (Ahlgen *et al*, 1995). During this process, among other proteins, the prostate predominantly secretes PSA (Lilja & Abrahamsson, 1988), which serves to cleave proteins secreted by the seminal vesicles (Lilja, 1985). Fluid secretions of the prostate make up 30% of semen (Huggins *et al*, 1942), and the prostate fluid contributes zinc and magnesium ions, as well as citric acid and prostatic acid phosphatase, and Beta-inhibin (Lilja & Abrahamsson, 1988; Carlson, 2014). The serine protease function of PSA and other kalikreinins (of which PSA is one) during ejaculation is essential for male fertility and reproductive success (Verze *et al*, 2016).

## 2.2. Prostate Cancer

### 2.2.1. Epidemiology

The first reported histological diagnosis of (PC) occurred in 1853, with it then being “a very rare disease” (Adams, 1853). PC incidence has since risen to become the most common cancer of men in developed countries (Ferlay *et al*, 2015). In Finland between 2010 and 2014, PC diagnoses

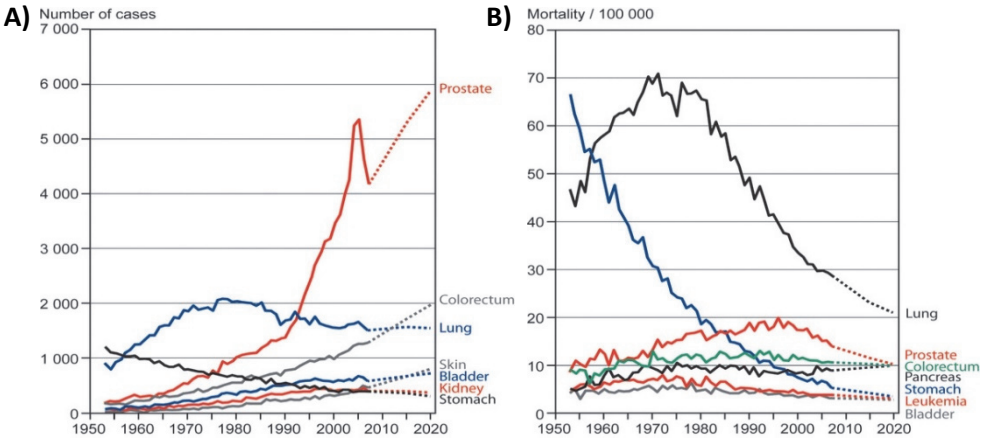


*Figure 2.* Incidence (A) and mortality (B) of male cancers in Finland. (Cancer in Finland, 2006) (Reprinted with permission, 25022017)

represented 30.6% of all new cancer diagnoses in men (Figure 2A) (Engholm *et al*, 2017). In the United States, approximately 161,130 new PC diagnoses were made in 2016, totaling 19% of all new male cancer diagnoses (Siegel *et al*, 2017). Despite its high incidence, approximately only 15% of men diagnosed with PC in Finland will die due to disease (Figure 2B) (Engholm *et al*, 2017).

## 2.2.2. Risk factors

The exact etiology of the development of PC is unknown, and many risk factors have been examined in epidemiological studies. Age is a known risk factor for PC incidence as work by Sakr et al, demonstrated in autopsy studies of men at different ages, an increased likelihood of PC detection with every increase in age by decade (Sakr *et al*, 1994).

### 2.2.2.1 Heredity and Genetics

Studies utilizing cancer registries have shown a familial component of PC risk. The hazard ratio of diagnosis of PC in males ranges from 2.1 to 23, depending on the relation and number of relatives affected (Figure 3) (Hemminki, 2012). Brothers of men diagnosed with Gleason 8-10 PC are themselves also more likely to be diagnosed with Gleason 8-10 PC (Jansson *et al*, 2012). In a study of twins from Sweden, Denmark, and Finland, 42% of cases were linked to heredity (Lichtenstein *et al*, 2000). Given that recent studies have shown that rare-subpopulations of PC patients present with germline mutations in certain known genes (Castro *et al*, 2013), a strong consensus statement has called for testing of *HOXB13* in all patients with a suspicion of hereditary PC, as well as *BRCA2* and *ATM* in advanced PC patients (Giri *et al*, 2018) given that novel targeted therapies may be effective in treating these PCs (Mateo *et al*, 2015).

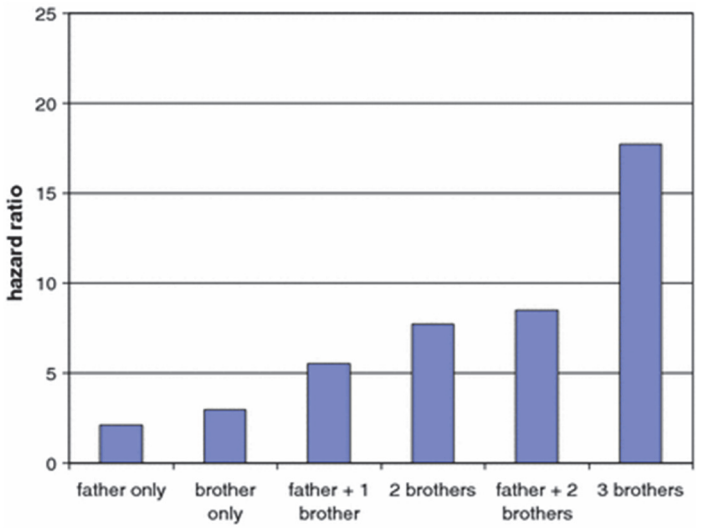


*Figure 3.* Hazard ratios for diagnosis of prostate cancer based according to the number of affect*ed relatives.* As adapted from (Hemminki, 2012) (Reprinted with permission, 21022017)

## 2.2.2.2 Other Factors

Other studies have shown the role of environmental and lifestyle risk factors in the development of PC. A prospective Finnish twin cohort study with 30 year follow up time, showed that increased rates of alcohol consumption was associated with increased risk of PC (Dickerman *et al*, 2016). Tobacco use was analyzed in a recent meta-analysis, which concluded that a modest, but statistically significant, association exists between tobacco use and PC incidence (Islami *et al*, 2014). Studies of Japanese males immigrants to the US, showed higher incidence of PC than non-migrants, and that their descendants similarly acquired the incidence profile of the US, indicating that lifestyle and environmental factors play some role in the etiology of PC (Shimizu ' *et al*, 1991).

In a prospective cohort, there was a positive association between consumption of red meat and PC (Giovannucci *et al*, 1993). A recent systematic review and meta-analysis showed a significant association between vertex-pattern androgenetic alopecia (also known as male-pattern baldness) and risk of PC diagnosis, but the same study found no association between any-pattern baldness and PC (Amoretti *et al*, 2013). A large scale case-control study assessed testosterone and other androgens with regards to risk of developing PC, and found no significant

association between testosterone levels and PC incidence (Severi *et al*, 2006).

## 2.3. Diagnosis of Prostate Cancer

### 2.3.1 PSA

Prostate-Specific Antigen (PSA) is a protein expressed mostly by the prostate. Discovered initially in 1960, it's biological and clinical characterization was carried out by a number of researchers between 1960 and 1980 (Rao *et al*, 2007). Subsequently, the US Federal Drug Administration approved the measurement of serum PSA to monitor PC status after primary therapy (De Angelis *et al*, 2007).

Efforts then turned to evaluating the use of PSA in staging and detection of PC, with a seminal report by Catalona et al. demonstrating that serum PSA screening, in combination with digital rectal examination (DRE) performed better than DRE alone in the detection of localized disease (Catalona *et al*, 1991).

The use of PSA in screening for PC is controversial. The use of PSA-screening for PC began in 1988, and increased exponentially, leading to an increase in incidence of diagnosed PC (Figure 2A) (Potosky *et al*, 1995). This contributed to a 50% decline in PC-specific mortality (Hu *et al*, 2016), however, as PSA may be elevated in patients without PC (Stamey *et al*, 1987), the specificity of PSA-screening remained in question. In 2008, the U.S. Preventive Services Task Force (USPSTF) came out against PSA-screening for men aged 75 and older (Committee & LS, 2008). In March 2009, the results of the US Randomized Prostate-Cancer Screening Trial (Andriole *et al*, 2009) and the European Randomized Study of Screening for Prostate Cancer (Schröder *et al*, 2009a) (of which 49.5% of patients were Finns) were released, which taken together, initially showed mixed results with regard to the effect of PSA screening on PC-specific mortality (Eckersberger *et al*, 2009). These results led to the USPSTF in 2012 to further recommend against PSA-screening for men of all ages (Moyer, 2012). However, recent analyses of both studies together, with longer term follow-up, suggest that ERSPC and PLCO both show that PSA-screening reduces PC mortality (Tsodikov *et al*, 2017). While it is well established now that PSA screening can reduce PC mortality (Schröder *et al*, 2014), it is also evident that PSA screening induces overdiagnosis of clinically insignificant PC. In fact, the relationship between PC mortality reduction and overdetection is almost linear

(Auvinen *et al*, 2016); as the PSA threshold is lowered, mortality reduction increases but at the cost of increased overdiagnosis. The current care guidelines in Finland (Suomalaisen Lääkäriseuran Duodecimin ja Suomen Urologiyhdistys ry:n asettama työryhmä., 2014) and in most other western countries besides recommend against routine PSA-screening for diagnosis of PC. As mentioned previously, the draft USPSTF recommendations on PSA-screening (USPSTF, 2017), will likely lead to increased PC incidence in the United States. Ideal screening would identify patients with, or at risk to develop, clinically adverse disease as early as possible, while in concomitantly identifying patients who are not at risk of developing adverse disease within their lifetime.

### 2.3.2 Prostate Biopsy

The development of the transrectal ultrasound (TRUS) guided sextant biopsy procedure in the late 1980's (Ragde *et al*, 1988; Hodge *et al*, 1989) led to its increased use in the diagnosis of PC (Potosky *et al*, 1995). Sextant biopsies were later shown to undersample clinically relevant lesions, as 24% of patients who underwent a second set of TRUS sextant biopsies after initial benign findings, had clinically relevant PC (San Francisco *et al*, 2003).

#### 2.3.2.1 Systematic Biopsy

Concurrently, other studies showed an increased ability of bi-sextant biopsies to detect PC as compared to sextant biopsy procedure (Djavan *et al*, 2000; Gore *et al*, 2001). These findings led to the development of the first active surveillance protocols, under which men with biopsy-confirmed indolent PC are directed to undergo systematic repeat biopsies in order to avoid or delay active treatment (Carter *et al*, 2002; Choo *et al*, 2002). AS will be further described in section 2.4.1.1. Due to the random nature of the TRUS-guided biopsy procedure, there is a known sampling error which can miss clinically relevant PC lesions (Schulte *et al*, 2008; Gallina *et al*, 2012; Washington *et al*, 2012; Iremashvili *et al*, 2013; Lahdensuo *et al*, 2015).

#### 2.3.2.2 Fusion Biopsy

Due to the aforementioned undersampling and error rate of systematic biopsies, there has been increasing interest in the development of multiparametric MRI (mpMRI) guided biopsy procedure (Garcia-Reyes *et*

*al*, 2015) which has recently been shown to have increased specificity for the detection of clinical significant PC (Ahmed *et al*, 2017). A novel magnetic resonance imaging and ultrasound fusion guided prostate biopsy (Fusion Bx), has been recently developed (Pinto *et al*, 2011). In brief, patients undergo prostatic MRI, and MRI imaging is electronically fused with an ultrasound-guided biopsy system, where the user can then target biopsies to lesions identified in MRI. Subsequent studies have shown that Fusion Bx can improve detection of clinically significant PC in men who have previous negative systematic biopsies (Vourganti *et al*, 2012; Sonn *et al*, 2014). Further studies have shown that Fusion Bx detects clinically adverse tumors at a higher rate than systematic Bx (Siddiqui *et al*, 2013, 2015). Given these findings, there is increased interest and research efforts into incorporating Fusion Bx into PC diagnosis.

### 2.3.3 Gleason Score and Grade Group

Per the EAU guidelines on PC, Gleason grade system is the strongest prognostic factor of PC (Mottet *et al*, 2017). The Gleason grading system was created in the 1960's (Figure 4) (Gleason, 1966). The most common histological pattern, termed Gleason primary grade pattern, is added to the second most common histological pattern, termed Gleason Secondary Score, and are added together to create the Gleason Score. A hallmark

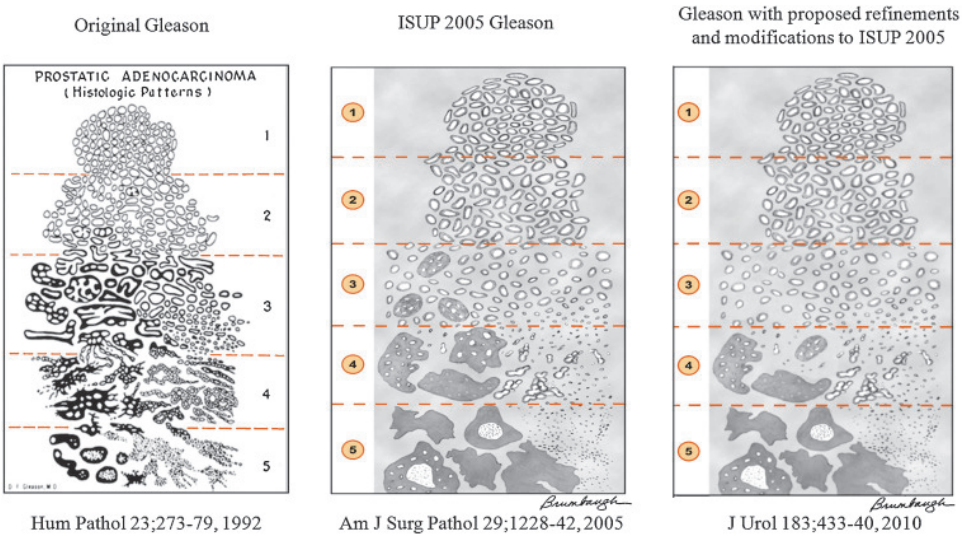


*Figure 4. Gleason grading over time. A) Original Gleason score, B) ISUP 2005, and C) 2010 ISUP refinements. (Reprinted with permission from* (Brimo *et al*, 2013)*,(Permission obtained 22022017)*

distinguishing benign prostate from adenocarcinoma is the presence of the basal cell layer (Figure 5A). Currently assigned Gleason Patterns for PC begin with well differentiated prostate glands lacking a basal cell layer in Gleason Pattern 3 (Figure 5B). As PC progresses, the glands become poorly differentiated in Gleason Pattern 4 (Figure 5C) to losing all glandular structure in Gleason Pattern 5 (Figure 5D).

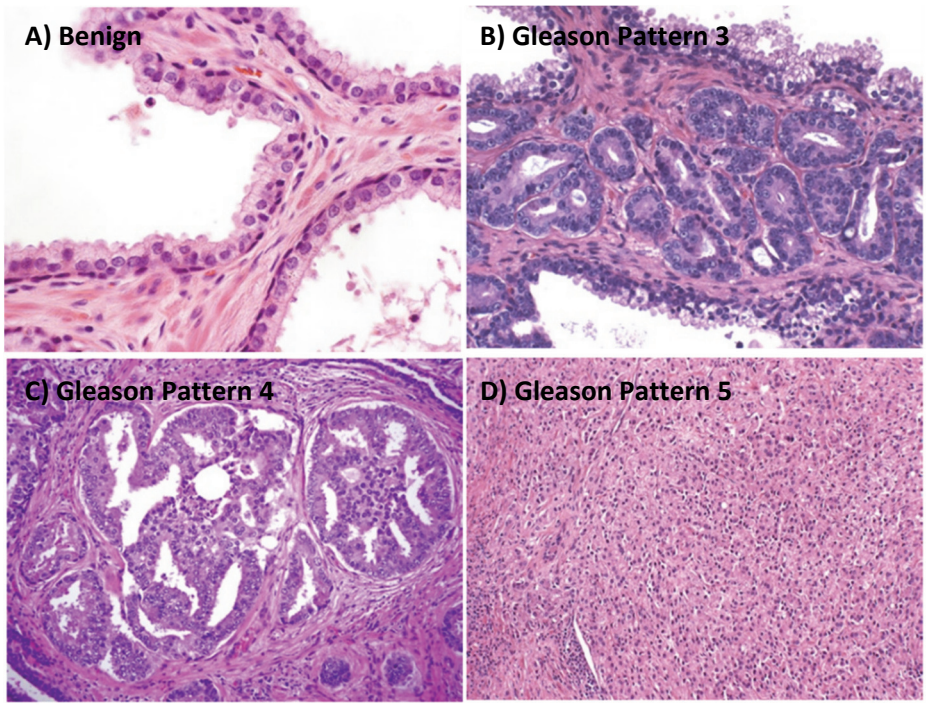


*Figure 5. Prostate Histology. A) Benign prostate, note the presence of basal cells, B) Gleason Pattern 3, C) Gleason Pattern 4, D) Gleason Pattern 5. Reprinted with permission from (Maclennan & Cheng, 2010) (Permission granted 17022018).*

In 2005, the system was revised to match modern practice, in which Gleason Pattern 1&2 are not assigned in clinical practice to needle biopsies (Figure 4B) (Epstein *et al*, 2005). Given the 2005 revisions to the Gleason scoring system, discussions subsequently commenced regarding cribriform gland scoring, which were originally defined as either cribriform Gleason pattern 3 or 4. At the 2010 ISUP meeting, discussion highlighted the fact that cribriform Gleason pattern 3 was rarely assigned in clinical practice, interobserver reproducibility was low, and that

cribriform Gleason pattern 3 almost always occurred in parallel with Gleason pattern 4. This lead to further revisions of the Gleason scoring system wherein cribriform pattern was revised to Gleason pattern 4 only (previously could also be assigned pattern 3) (Figure 4C) (Epstein, 2010). Despite these changes being well understood and received by treating clinicians, the Gleason grade scale was not intuitive for patients to interpret; the lowest assigned pattern in routine clinical practice was Gleason score 6, yet, patients often interpret this as being in the middle of a tier grades from 2-10, leading to patient anxiety and overtreatment. This lead to the creation of the PC Grade group, where Gleason grades were re-scaled into a scale of 1-5 (Epstein *et al*, 2016). In clinical practice, patients are risk-classified into low, intermediate, and high-risk groups, with patients having GG1 PC being classified as low-risk, and patients with GG 4-5 PC being classified as high-risk. Please see Table 1 for a comparison of Gleason score and contemporary Grade Group.

**TABLE 1.** Gleason Score vs Grade Group.
Modified and reprinted with permission from Study IV (10.02.2018)

| Gleason Score | Grade Group |
|---|---|
| ≤6 | 1 |
| 3+4 | 2 |
| 4+3 | 3 |
| 4+4<br>5+3<br>3+5 | 4 |
| 4+5<br>5+4<br>5+5 | 5 |

## 2.3.4 Stage and TNM

PC is commonly staged using the Union for International Cancer Control Prostate Cancer TNM classification system (Table 2) (Brierley *et al*, 2016). PCs are assigned a clinical TNM stage at diagnosis, whereas pathological TNM stage (pTNM) is given after pathologist review of surgical prostate

samples. Organ-confined PC is assigned T1 or T2, and locally advanced PC is assigned either T3 or T4.

**Table 2**. Modified, reprinted with permission from Brierley et al, 2016 (permission obtained 14032017).

| | | |
|---|---|---|
| T - Primary Tumor | | |
| Tx | Primary tumour cannot be assessed | |
| T0 | No evidence of primary tumour | |
| T1 | Clinically inapparent tumour not palpable or visible by imaging | |
| | T1a | Tumour incidental histological finding in 5% or less of tissue resected |
| | T1b | Tumour incidental histological finding in more than 5% of tissue resected |
| | T1c | Tumour identified by needle biopsy (e.g. because of elevated prostate-specific antigen (PSA) level) |
| T2 | Tumour confined within the prostate | |
| | T2a | Tumour involves one half of one lobe or less |
| | T2b | Tumour involves more than one half of one lobe, but not both lobes |
| | T2c | Tumour involves both lobes |
| T3 | Tumour extends through the prostatic capsule | |
| | T3a | Extracapasular extension (unilateral or bilateral) including microscopic bladder neck involvement |
| | T3b | Tumour invades seminal vesicles |
| T4 | Tumour is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum, levator muscles, and/or pelvic wall | |
| N - Regional Lymph Nodes | | |
| NX | Regional lymph nodes cannot be assessed | |
| N0 | No regional lymph node metastasis | |
| N1 | Regional lymph node metastasis | |
| M - Distant Metatstases | | |
| MX | Distant metastasis cannot be assessed | |
| M0 | No distant metastasis | |
| M1 | Distant metastasis | |
| | M1a | Non-regional lymph node(s) |
| | M1b | Bone(s) |
| | M1c | Other site(s) |

### 2.3.5 Prostate MRI

The use of multiparametric MRI (mpMRI) in the diagnosis and staging of PC has increased in the past 5-10 years due to advances in imaging techniques. The Prostate Imaging – Reporting and Data System (PIRADS) has increased in popularity for proper recording and reporting of mpMRI findings (Figure 6) (Weinreb *et al*, 2016). The recently published PROMIS trial showed that mpMRI is more sensitive that TRUS biopsy in predicting clinically adverse PCs (Ahmed *et al*, 2017). While initial results

are promising, further work is needed, as mpMRI can miss up to 20% of index lesions and other high grade lesions (Le *et al*, 2015). In addition, in the United States, access to MRI outside of academic medical centers is low (Leake *et al*, 2014). Lastly, a recent study showed that radiologists

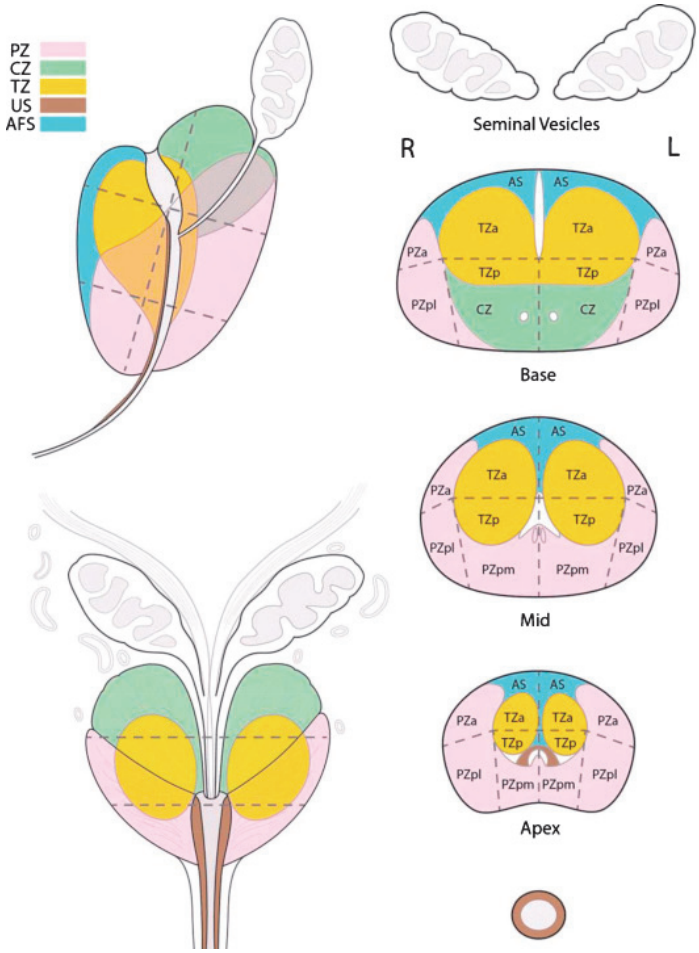


*Figure 6.* Sector map of the PIRADS v2 reporting system. Reprinted with permission from (Garcia-Reyes et al, 2015) (permission obtained 25.01.2018).

were an independent predictor of detecting clinically adverse PC in mpMRI (Le *et al*, 2015). Research into the specific incorporation of mpMRI into clinical routine is an area of active research, and mpMRI will likely be incorporated into clinical routines in the future.

## 2.4 Treatment of Prostate Cancer

Treatment of PC can be divided into palliative treatment of symptoms without curative intent, and treatment of PC with curative intent (Figure 7). The following sections will focus on the latter category.

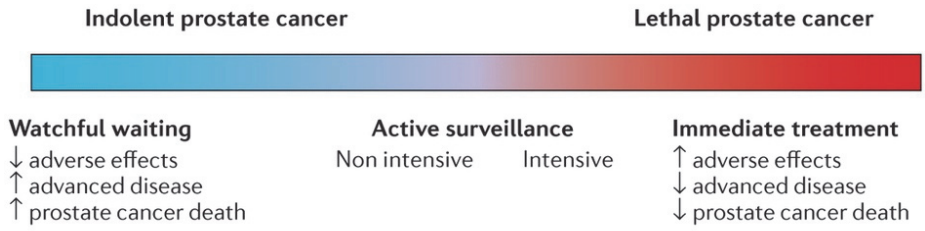


*Figure 7. Treatment of indolent to lethal prostate cancer. Modified with permission from* (Tosoian *et al*, 2016) *(*Permission granted *14032017).*

### 2.4.1 Primary Treatments with Curative Intent

#### 2.4.1.1 Active Surveillance

PSA-screening has led to a decrease in PC-specific Mortality (Schröder *et al*, 2014). PSA-screening, however, leads to over-diagnosis of indolent PC (Etzioni *et al*, 2002) resulting in overtreatment. An alternative option to radical treatment in patients with favorable-risk disease is AS.

AS cohorts differ worldwide in their inclusion criteria, however, most AS protocols feature systematic monitoring of PSA-metrics and histopathological parameters from repeat prostate biopsies (Klotz *et al*, 2010; Tosoian *et al*, 2011; Bul *et al*, 2013). AS has been highly utilized in the Nordics, especially Sweden (Loeb *et al*, 2015, 2017), and until relatively recently, underutilized in the United States (Cooperberg *et al*, 2011a). In the PRIAS trial, 52% and 72% of men discontinue AS at 5 years and 10 years follow-up respectively (Bokhorst *et al*, 2016). AS 10 year disease-specific survival rates for AS are high, and range in the 93-98.1% range (Klotz *et al*, 2015; Tosoian *et al*, 2015). A recent randomized clinical trial of active monitoring, radiotherapy, and radical prostatectomy in patients with T1 or T2 PC showed that there was no difference in survival between the different study arms (Hamdy *et al*, 2016). Figure 8 shows the clinical decision tree for PRIAS AS trial follow up. The PRIAS trial is

currently the largest prospective AS cohort in the world with more than 7000 recruited patients.

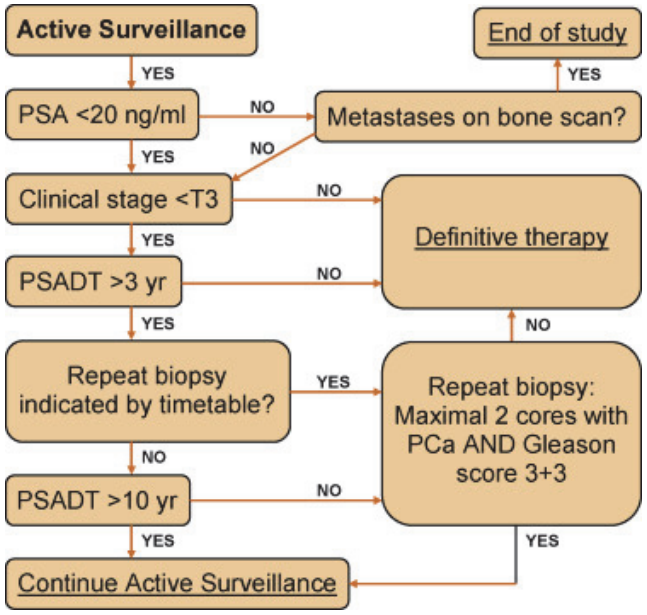


*Figure 8.* Follow-up criteria decision tree for the PRIAS cohort. Note that the updated PRIAS criteria no longer include PSADT > 3 years as a trigger. PSA = prostate-specific antigen; PSADT = PSA doubling time; PCa = prostate cancer. Reprinted with permission from (Van Den Bergh *et al*, 2007) (Permission granted 14032017).

Many studies, including the PRIAS trial (of which HUS is a member), initially utilized PSA-kinetics in AS criteria (Bul *et al*, 2013). a recent study showed that such metrics do not reliably predict adverse prostate pathology (Ross *et al*, 2010). In the PRIAS trial, men that discontinued due to PSA-DT<3-years only and underwent radical prostatectomy, almost 50% had clinically insignificant prostate cancer. Subsequently, PRIAS criteria were revised such, that a PSA-doubling time of less than 3 years no longer automatically triggers treatment and use of PSA kinetics in AS as a trigger for treatment is decreasing.

A recent study of 131 patients showed that a 3 gene molecular signature, consisting of *FGFR1*, *PMP22*, and *CDKN1A* significantly predicted AS outcomes (Irshad *et al*, 2013). Another recent study of n=265 Danish AS patients found that ERG status, as determined by immunohistochemistry, could be used to predict AS outcomes (Berg *et al*, 2014).

### 2.4.1.2 Radical Prostatectomy

The modern radical prostatectomy procedure entails the removal of the prostate gland, seminal vesicles, surrounding tissue to ensure negative surgical margins, and is often performed in conjunction with removal of the pelvic lymph nodes (Mottet *et al*, 2017). The first prostatectomy in recorded history was performed in 1885 (Thorndike, 1903). Subsequently, the open, radical, perineal prostatectomy procedure, whose derivative is still in use in modern practice, was developed in 1904 (Young, 1905). In 1945, the first radical retropubic prostatectomy procedure was reported (Millin, 1945). In 1991, the first laparoscopic radical prostatectomy (LRP)

*Figure 9. Robotic-assisted laprascopic prostatectomy at Peijas Hospital, HUS Urology. Image taken by author, used with permission.*

was performed (Schuessler *et al*, 1997). In 2001, the first Robotic-Assisted Laparoscopic Prostatectomy (RALP) was performed, and Figure 9 displays the operating room setup for the RALP procedure (Abbou *et al*, 2001). A study of patients undergoing LRP or RALP as compared to patients undergoing traditional retropubic RP (RRP) found, that patients in the former group had shorter hospital stays, fewer surgical complications, but experienced greater incontinence, erectile dysfunction, and other genitourinary issues (Hu *et al*, 2009). The recent non-randomized LAPPRO trial from Sweden, however, showed decreased

rates of erectile dysfunction for patients undergoing RALP as compared to RRP, and no difference in rates of urinary incontinence or positive surgical margins (Haglind *et al*, 2015).

In a randomized clinical trial of watchful waiting or radical prostatectomy with long follow up time, radical prostatectomy was shown to be effective in reducing PC-specific mortality (Bill-Axelson *et al*, 2014). Recent randomized trial comparing active monitoring, radical prostatectomy and RT could not demonstrate survival differences between treatment arms at 10 years (Hamdy *et al*, 2016). Many retrospective cohort analyses show benefit for radical prostatectomy over RT (Zelefsky *et al*, 2010). In a recent, large scale retrospective analysis of radical prostatectomy vs RT in Sweden, there were negligible differences in outcome of high-risk PC comparing RT and radical prostatectomy (Robinson *et al*, 2017). Despite it's well demonstrated utility in reducing PC progression and PC-specific mortality, radical prostatectomy is known to significantly affect patient's quality of life after treatment, including urinary incontinence, bowel leakage, erectile dysfunction (Litwin *et al*, 1995; Sanda *et al*, 2008; Mols *et al*, 2009).

#### 2.4.1.3 Radiotherapy

Radiotherapy (RT) of PC is another common treatment of primary PC, with the first application of RT for PC being reported in the early 1900's (Minet, 1909; Pastean, O., Degrais, 1913; Young, 1917).

Modern RT treatment of PC is primarily delivered via two different techniques, brakytherapy or external beam radiotherapy (EBRT) (Figure 10A). The modern brakytherapy method, first described in 1983 (Holm *et al*, 1983), involves the insertion of 'seeds' made of a radioactive isotope, commonly Iodine-125 or Caesium-131 (Lemoigne & Caner, 2009), into the prostate (Figure 10B). Radiation via BT can be delivered either at a low dose rate (LDR) or high dose rate (HDR) with LDR-BT being most commonly used in the treatment of PC (Skowronek, 2013). EBRT, in contrast, delivers RT via a beam from an external source to a targeted location (Figure 10B).

Primary PC patients undergoing BT typically suffer less side effects compared to EBRT and radical prostatectomy (Chen *et al*, 2009), but suffer from a poorer median disease-free survival (Kibel *et al*, 2012). RT is often used as second line or salvage therapy in patients treated with

prostatectomy, with approximately half of all salvage RT patients responding to therapy (Stephenson *et al*, 2007).

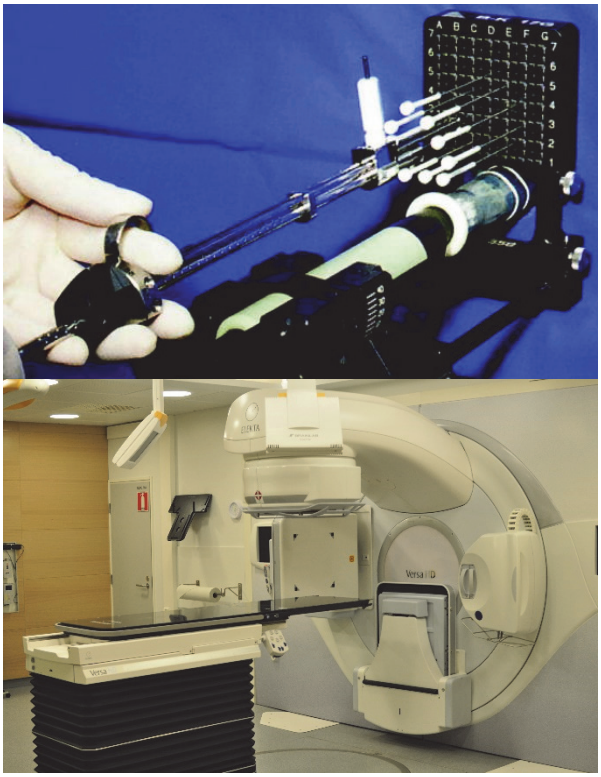

*Figure 10. A) Picture of the method by which brakytherapy seeds are implanted into the prostate (Battermann, 2013) (Reprinted with permission 26062017). B) Picture of the HYKS comprehensive cancer Versa HD™ External Beam Radiotherapy (Photo taken by author with permission, 10.08.2017).*

Again, a recent Phase III prospective ProtecT trial in patients diagnosed with localized PC (cT1-2), which randomized patients, to active monitoring, RT, and radical prostatectomy, found no difference in 10 year PC-specific mortality (Hamdy *et al*, 2016). Many retrospective and prospective observational studies have compared RT to radical prostatectomy in patients with high risk disease, finding that patients undergoing radical prostatectomy have a better survival profile (Albertsen *et al*, 2007; Tewari *et al*, 2007; Lee *et al*, 2014; Sooriakumaran *et al*, 2014). To date, there have been no randomized clinical trials comparing RT and radical prostatectomy in a higher risk patient cohort, as ¾ of patients in the ProtecT trial were low-risk patients. The SPCG-15 trial is

currently enrolling patients to randomize patients with pT3 PC to RT or radical prostatectomy (ClinicalTrials.gov).

### 2.4.2 Outcomes

Obtaining long term, clinically relevant PC outcome data accurately, systematically, and efficiently, is a challenge world-wide. Finland has a Personal Identity Number (PIN) which is somewhat analogous to Social Security Number (SSN) used in the United States. In Finland, PIN contains a 6 digit date of birth, a single character century identifier and a 4 character unique identifier. One crucial distinction between the healthcare systems of the United States and Finland, is the ubiquity in the Finnish healthcare system to utilize PIN as a patient specific identifier. Specifically, this means that all Electronic Health Records (EHR) at the hospital level as well as national registry data can be linked using PIN. Qualified investigators can apply for the right to use the registry data. Additionally, qualified clinical investigators, with IRB approval, can link hospital level structured and unstructured EHR data. The National Institute for Health and Welfare (THL) can provide researchers an approval to join data from different population level and national health care registries. Furthermore, all pathology centers across Finland utilize sample specific identifiers that can be linked to PIN, which can be further linked to the aforementioned EHR.

Due to the ease of identification of patient-specific records through the unified Electronic Health Record (EHR) use of PIN, researchers are able to characterize not only prostate cancer-specific mortality or any cause mortality, but other relevant outcomes identifying clinically relevant disease.

In summary, the Finnish healthcare system and biobank law, through use of PIN, allows for linking hospital health records, national registry data, and patient samples.

## 2.5. Biomarkers in Prostate Cancer

The human protein atlas study (Uhlén *et al*, 2015) recently reported that in transcriptomic analysis of the prostate, 73% (14,224) of all proteins are expressed in prostatic tissues. Given their focus in this dissertation, this section will begin with a discussion of Androgen Receptor, ERG, and PTEN (2.5.1, 2.5.2, and 2.5.3), with discussion of other clinically relevant biomarkers discussed in section 2.5.4 and onwards.

## 2.5.1 Androgen Receptor

Androgen receptor (AR) is a nuclear receptor (Lu *et al*, 2006) that has an important role in both development of the prostate (Hayward *et al*, 1996; Roy *et al*, 1999), and PC (Scher & Sawyers, 2005). AR is a DNA-binding transcription factor (Mooradian *et al*, 1987), encoded by the X-chromosomal gene *AR* (Chang *et al*, 1988; Trapman *et al*, 1988). The androgens testosterone and 5α-dihydrotestosterone act biologically through binding AR (Figures 11, 12) (Heinlein & Chang, 2004; Lonergan & Tindall, 2011). In prostate glands, most prostate cells express some level of AR while only luminal cells are actually dependent on AR expression (Maitland, 2008; Maitland *et al*, 2011; Packer & Maitland, 2016). Secondary therapies of PC focus on androgen deprivation by either chemical (Klotz, 2006) or surgical castration (Huggins *et al*, 1941). Androgen deprivation therapy (ADT), however, is not curative, and PC progresses to a castration-resistant state. The second generation anti-androgen enzalutamide (Cabot *et al*, 2012) has recently been shown in

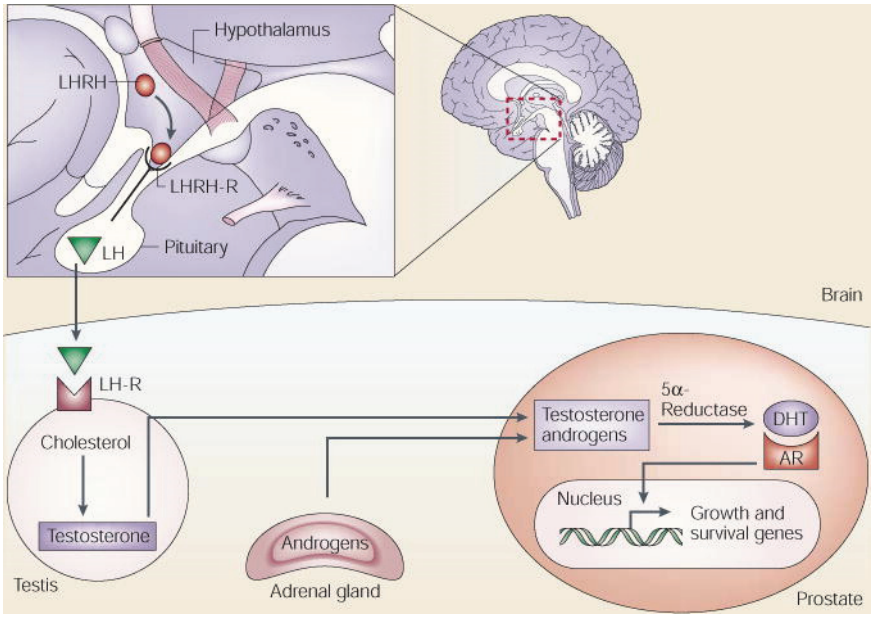


*Figure 11.* Androgen Action and Production (Denmeade & Isaacs, 2002). (Reused with permission, 24062017).

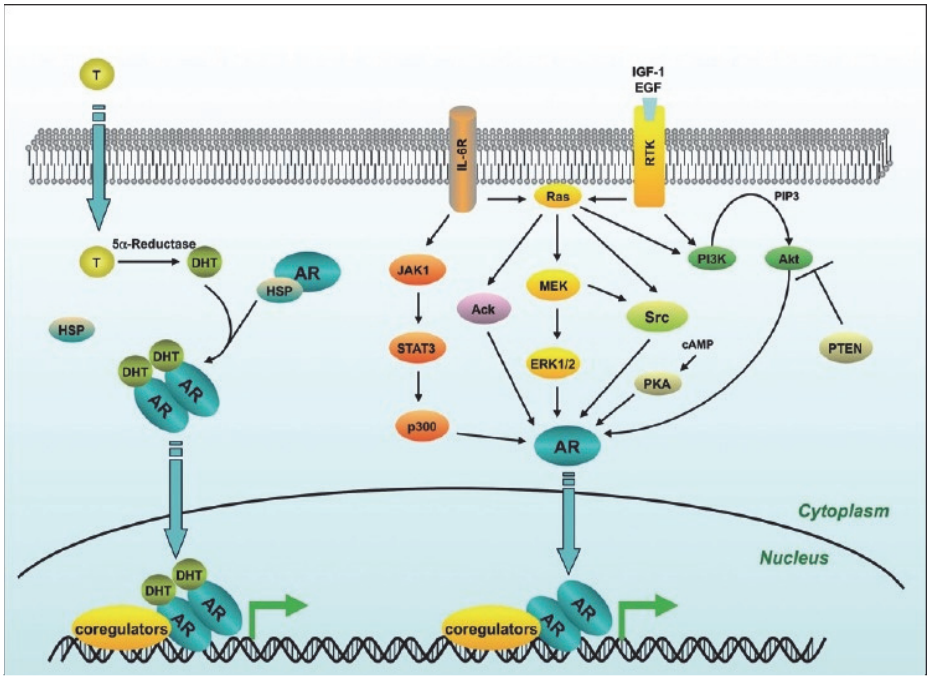


*Figure 12. Androgen receptor signaling. Reused with permission under a Creative Commons Attribution-Noncommercial-ShareAlike 3.0 Unported license, accessed 26022017 (Lonergan & Tindall, 2011)*

Phase III clinical trials to be effective in increasing median survival time in mCRPC patients. AR splice variants have been implicated in being involved in resistance to abiraterone and enzalutamide treatment (Antonarakis *et al*, 2014; Welti *et al*, 2016). A Phase III trial of a novel, next generation, anti-androgen, ARN-509 (also known as Apalutamide), was initiated in 2014 (Smith *et al*, 2014). Initial results reported in February 2018 showed that ARN-509 reduced metastatic progression as compared to placebo in patients with non-metastatic CRPC (Small *et al*, 2018). A third novel antiandrogen, ODM-201 (Darolutamide) has also shown promise in the phase I-II ARADES trial (Fizazi *et al*, 2014) and is currently being evaluated in a phase III trial (ARAMIS) in M0 CRPC PC (Fizazi *et al*, 2016).

## 2.5.2 ERG

ERG is an ETS (erythroblast transformation-specific) family transcription factor, encoded by the *ERG* gene (Reddy *et al*, 1987). ERG commonly forms gene fusions with TMPRSS2, and a seminal paper found that the ERG:TMPRSS2 gene fusion has been found to occur in 40-70% of PC (Tomlins *et al*, 2005), leading to increased investigation of the gene fusion

in PC. Genetic aberrations of ERG alone are not sufficient to initiate PC (Tomlins *et al*, 2008; Carver *et al*, 2009a; King *et al*, 2009). ERG has been shown to disrupt AR signaling (Yu *et al*, 2010). The association of ERG fusion with clinical outcomes in PC is controversial. While studies have shown ERG fusion positivity to be associated with PC outcomes (Demichelis *et al*, 2007; Yoshimoto *et al*, 2008a; Grupp *et al*, 2013; Berg *et al*, 2014; Fontugne *et al*, 2014), others have shown ERG fusion positivity not to be associated with PC outcome (Reid *et al*, 2010; Ahearn *et al*, 2015). ERG status, in combination with PTEN status (Figure 13) (Squire, 2009), has been shown to be predictive of PC outcomes (Carver *et al*, 2009b; Reid *et al*, 2010; Chen *et al*, 2013; Ahearn *et al*, 2015).

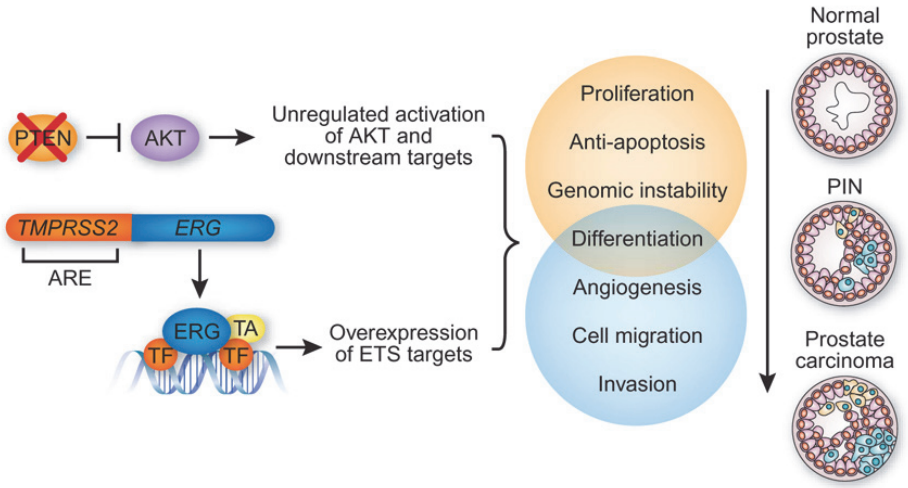


*Figure 13. TMPRSS2:ERG gene fusion and PTEN in PC progression. Reused with permission (Squire, 2009) (Permission granted 26022017).*

## 2.5.3 PTEN

PTEN protein is encoded by the gene *PTEN* and is found on chromosome 10, location q23.21 (Steck *et al*, 1997a). *PTEN* has been identified as a tumor suppressor in a large number of cancer types. PTEN functions to dephosphorylate PIP3, generating PIP2 and SHIPs (Damen *et al*, 1996). PIP3 signals in the AKT pathway, and because of this signaling (Figure 14) (Phin *et al*, 2013), loss of PTEN function leads to increased PIP3 expression, which activates the AKT pathway. PTEN's function as a tumor suppressor hinges on this activity.

Deletions of the *PTEN* gene are the most common cause of loss of PTEN functionality (Cairns *et al*, 1997; Steck *et al*, 1997b), however, genomic

rearrangements have also been found to result in loss of PTEN function (Reid *et al*, 2012). Studies have shown that between 35-63% of PC's have PTEN loss (Feilotter *et al*, 1998; Suzuki *et al*, 1998; Müller *et al*, 2000). PTEN loss alone has been shown to be associated with PC outcomes (Reid *et al*, 2010; Chaux *et al*, 2012; Krohn *et al*, 2012; Cuzick *et al*, 2013; Barnett *et al*, 2014; Ahearn *et al*, 2015), however, status of PTEN expression, in combination with status of ERG expression, has been shown to increase predictive value of PC outcomes (Carver *et al*, 2009b; Reid *et al*, 2010; Chen *et al*, 2013; Ahearn *et al*, 2015).

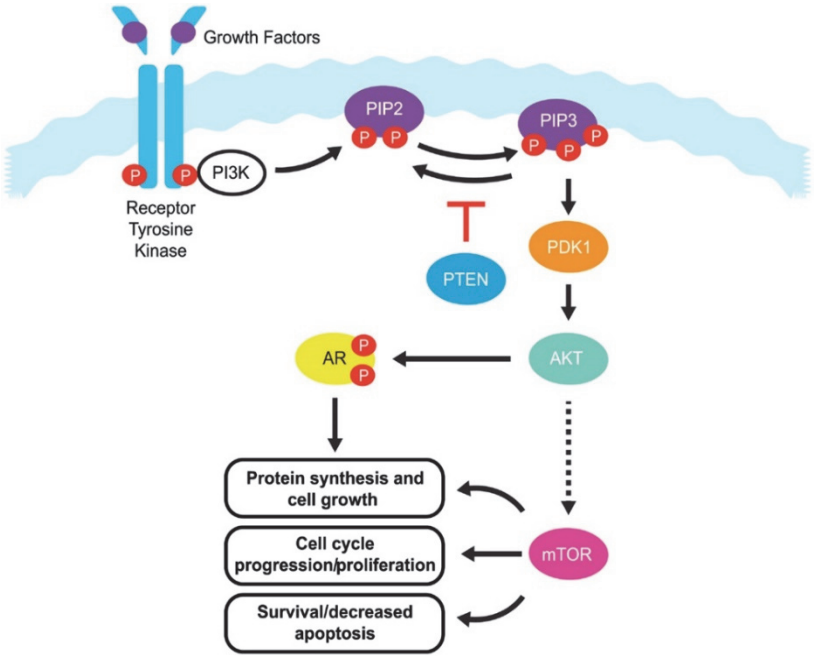


*Figure 14.* PI3K/PTEN/Atk signaling pathway. (Phin *et al*, 2013) (Reused with permission under a Creative Commons Attribution License (CC BY), accessed 26022017).

## 2.5.4 Other biomarkers

A biological marker (biomarker), as defined by the NIH Biomarkers Definitions Working Group, is "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Group, 2001). In the context of PC, Prensner et al. defined a broad framework definitions of seven biomarker types in the PC context (Prensner *et al*, 2012), please see their definitions in Table 3.

Table 3. Biomarker marker type and the clinical question addressed as defined by Presener et al., 2012

| Biomarker | Clinical Question |
|---|---|
| Disease disposition | What is a patient's risk of developing cancer in the future? |
| Screening | Does earlier detection of patients with cancer decrease mortality? |
| Diagnostic | Who has cancer? What is the grade of the cancer? |
| Prognostic | What clinical outcome is most likely if therapy is not administered? |
| Predictive | Which therapy is most appropriate? |
| Monitoring | Is therapy effective? Does the patient's disease recur? |
| Pharmacogenomic | Do genetics predict response to therapy or the risk for adverse reaction to the prescribed therapeutic dose? |

Active research into PC biomarkers at all stages of clinical progression as well as extensive preclinical research is ongoing worldwide, however, in the context of this specific dissertation, the following section will focus on biomarkers in a translational and clinical aspect, specifically in screening, diagnosis, and prognosis of PC.

Interest in PC biomarker discovery and translation has focused primarily on blood, tissue, urine, and semen (Figure 15) (Velonas *et al*, 2013). Blood, urine, and semen offer relatively non-invasive methods to obtain patient-derived biomaterial for screening, potential diagnosis, and prognosis. The current EAU guidelines, however, state that "Definitive [PC] diagnosis depends on histopathological verification of adenocarcinoma in prostate biopsy cores or specimens" (Mottet *et al*, 2017). In the absence of randomized trials comparing tissue-based diagnostics and diagnostics regimens derived from blood, urine, or semen, histopathological evaluation of prostatic tissue will continue to be the utilized as the gold standard of evaluation PC patient diagnosis. In the PC biomarker context, this provides an opportunity to translate tissue-based diagnostic and prognostic signatures into clinical context. Prostate biopsies are by nature invasive and are associated with co-morbidities (Lahdensuo *et al*, 2016a), including rare reports of mortality, however, in Finnish patients of the ERSPC trial, no excess mortality was reported due to Bx (Carlsson *et al*, 2011).

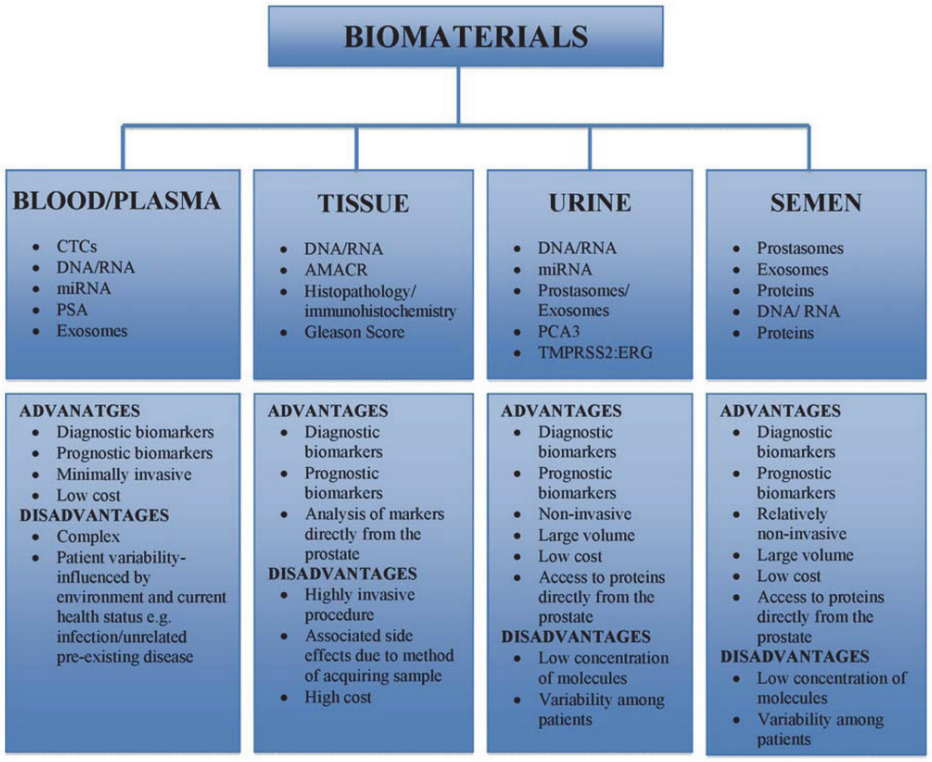


*Figure 15.* Potential sources of patient derived biological materials for PC biomarker discovery and translation. Reprinted with permission under a Creative Commons Attribution license V3.0 from Velonas et al., 2013, 11.**02**.2018.

## Biomarkers in the screening of PC

As discussed more extensively in section 2.3.1, PSA has been utilized for over 25 years as a biomarker for the screening of PC. While PSA screening has been shown in randomized clinical trials to reduce PC mortality (Andriole *et al*, 2009; Schröder *et al*, 2009b; Tsodikov *et al*, 2017), it comes at the cost of overdiagnosis and overtreatment of indolent low-risk PCs.

In addition to PSA, a number of other biomarkers have been proposed for use in PC screening. The recently announced Finnish ProScreen trial (Auvinen *et al*, 2017) will soon be enrolling patients in a randomized screening trial of the 4Kscore (Parekh *et al*, 2015). The 4Kscore includes 3 PSA measures, as well as hK2 (kallikrein-related peptidase-2), and has been shown to reduce the number Bx needed to be taken, while detecting approximately 90% of all GG 2 and higher PC's (Carlsson *et al*, 2013; Bryant *et al*, 2015; Braun *et al*, 2016). Similarly, the Prostate Health Index,

is another kallikrein based biomarker that has been proposed for use in PC screening (Fossati *et al*, 2015; Nordström *et al*, 2015). Additionally, the Stockholm-3 prospective study tested a model containing PSA metrics, single-nucleotide polymorphisms, and other clinical variables in a large scale population setting, finding that STHLM3 outperformed PSA in detecting clinically adverse PC (Grönberg *et al*, 2015).

A number of non-PSA based biomarkers for screening of PC have also been proposed. PCA3, a noncoding RNA, has been proposed as a prostate-specific screening biomarker (Hessels *et al*, 2003), and a recent study showed that urinary testing of PCA3 and TMPRSS2:ERG was able to detect GG2 and higher PCs while also reducing the number of Bx (Sanda *et al*, 2017).

Lastly, other fluid based biomarkers such as extracellular vesicles have been proposed for PC screening (Duijvesz *et al*, 2011; Puhka *et al*, 2017).

Biomarkers in the diagnosis of PC

A number of biomarkers have been investigated and are used to aid the differential diagnosis of PC. AMACR (α-Methylacyl Coenzyme A Racemase) was first identified by Rubin et al. as a tissue-based biomarker that is associated with PC (Rubin *et al*, 2002). Almost all prostatic adenocarcinomas are AMACR positive (Moch, H., Humphrey, P.A., Ulbright, T.M., Reuter, 2016), and AMACR expression has been shown to be associated with biochemical recurrence and disease-specific survival (Rubin *et al*, 2005). Contemporary studies showed that another protein, p63, is highly specific for prostatic basal cells (Parsons *et al*, 2001), and is relevant for PC diagnosis as loss of the prostatic basal cell layer is considered a hallmark of progression to PC. Subsequent work showed that antibodies specific for high weight molecular keratins (34betaE12) can also be used to help elucidate the basal cell layer (Shah *et al*, 2002). These results have led to guideline recommendations (Moch, H., Humphrey, P.A., Ulbright, T.M., Reuter, 2016) of the use of a “triple stain” of AMACR/p63/34betaE12 (Figure 16) in the diagnosis of PC.

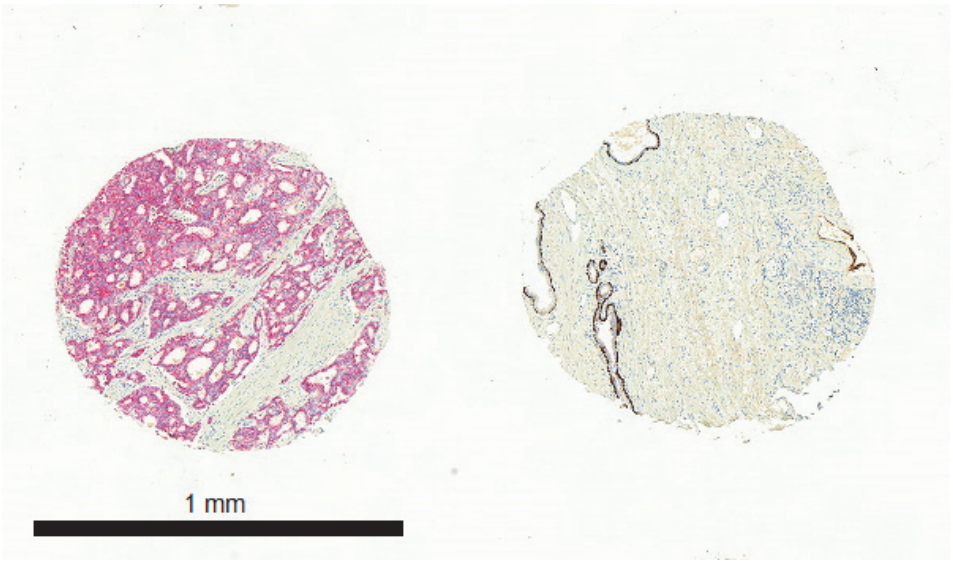


*Figure 16*. Triple staining for AMACR, p63, and cytokeratins 5/6 from tissue microarray spots of patients from Study III (unpublished, reused with permission 11.02.2018).

Other markers commonly used in HUS Pathology, and other centers, for diagnosis of PC include markers for differential diagnosis of neuroendocrine PC (NEPC), a rare and aggressive subvariant of PC. NEPCs feature distinct molecular hallmarks, including differential expression of p56, synaptophysin, and chromogranin (Ro *et al*, 1987; Wang & Epstein, 2008; Moch, H., Humphrey, P.A., Ulbright, T.M., Reuter, 2016), allowing IHC of these markers to be utilized.

Biomarkers in the prognosis of PC

In the landscape of localized PC, in addition to the previously described AR, ERG, and PTEN, a number of other biomarkers have been investigated for the role in PC carcinogenesis, progression, and prognosis. Given that high AR expression in primary PC is associated with poor outcomes, studies of molecular regulation of AR have been of interest. Among putative AR regulators, FoxA1, a forkhead protein, has been studied (Sahu *et al*, 2011). Sahu et al showed that low PC FoxA1 expression, even in a high AR background, was associated with good prognosis. Of note, Sahu et al's study was performed on some of the same patient material as analyzed in Study III. Concomitantly to the study published by Sahu et al., Barbieri et al. published a study shortly thereafter where they analyzed 112 patients primary PC's, and also found mutations in FoxA1, but also found that another gene, SPOP, was mutated in 13%

of all PC's (Barbieri *et al*, 2012). Interestingly, the authors found that the patients with SPOP mutations were much less likely to harbor ETS-fusion, suggesting PCs with SPOP mutations represent a distinct molecular subclass with regards to PC progression. A follow up study validated their finding in a much larger multicenter patient cohort of 720 PC patients, wherein 58 patients harbored SPOP mutations, and strikingly, only 2 also harbored ERG-rearrangements (Blattner *et al*, 2014). Their study, however, also reported that SPOP mutational status was not associated with biochemical recurrence.

There is an urgent unmet need for better clinical solutions to distinguish between clinically aggressive and indolent PCs. Interest and understanding of genomics in the context of PC at different stages of disease progression (Cancer Genome Atlas Research Network, 2015; Rubin, 2015; Rubin *et al*, 2016; Rubin & Demichelis, 2018) has led to academic (Zehir *et al*, 2017) and commercial development of genomic based tests for PC prognosis. Prolaris, a 31 gene signature panel, is a commercial transcriptome panel that has been proposed for use in diagnosing PC (Cuzick *et al*, 2011). The initial study of Prolaris focused on modelling biochemical recurrence, and later was validated in other cohorts with other outcomes. Decipher is another proposed commercial transcriptomic panel, featuring 22 genes (Erho *et al*, 2013), training their classifier on metastatic progression with 16.9 year follow-up. A recent validation study has shown that in 235 intermediate and high-risk PC patients with 6 years of follow-up, that Decipher remained an independent predictor of metastasis and prostate cancer-specific mortality (Nguyen *et al*, 2017) Another proposed reverse transcriptase-polymerase chain reason (RT-PCR) based panel, Oncotype Dx, has also be developed for PC prognostication (Knezevic *et al*, 2013). None of these tests have been compared comprehensively in the same patients' material. Additionally, many prognostic factors may predict surrogate outcomes but do not predict other clinically relevant outcomes of PC. Similarly, prognostic factors trained on certain outcomes, may not be as easily applied/translated to other stages of disease progression.

# 3. Aims of the studies

While primary therapies for localized disease are generally effective, there is an unmet clinical need for better risk-stratification. Current clinical treatments for low-risk disease result in over treating patients for a disease that is overwhelmingly unlikely to kill them during current normal lifespans. While this wouldn't necessarily be an issue, the current standard-of-care treatments themselves can cause significant co-morbidities, side-effects, and can even be lethal.

The specific aims of the studies were as follows:

1) To determine whether a novel predictor, the cumulative number of cancer positive locations (CCLO) during the diagnostic phase of AS, could predict poor outcomes of AS in a multi-center international cohort of AS patients.

2) To determine whether protein expression status of ERG and PTEN, as detected by immunohistochemistry, could be used to identify patients with higher risk disease at the time of inclusion into AS.

3) To determine whether ERG and PTEN protein expression, as assessed by immunohistochemistry, could stratify patients into different survival profiles in a multi-center patient cohort with long term follow-up and clinically relevant outcomes.

4) To determine whether the novel Grade Group system outperforms Gleason Scoring in a multi-center cohort with mortality outcomes and long term follow-up.

# 4. Materials and Methods

## 4.1 Study Populations (I-IV)

Study I:

For Study I, the study cohort was derived from three prospective AS cohorts, original totaling 609 patients from the Helsinki and Uusimaa Hospital District (HUS) PRIAS cohort, the University of Muenster PRIAS cohort, and the IEO AS cohort. After patient selection for inclusion criteria (below), the final pooled study cohort consisted of 380 patients from all three centers. For detailed Study I cohort demographics, please see Table 4.

Between January 2007 and November 2015, 316 patients were enrolled into the HUS PRIAS cohort. We queried all available PRIAS database clinical data and HUS medical record data from these patients. We then selected all patients who underwent diagnostic biopsy and confirmatory biopsy, and continued in AS, of which totaled 149 patients.

Between February 2005 – June 2016, 204 patients were enrolled into the IEO AS cohort. We queried all available AS medical record data from these patients. As IEO's AS inclusion criteria differed from PRIAS, notably by allowing the inclusion of patients with 3+4 disease, we excluded these patients from analysis. We then selected for all patients who underwent diagnostic biopsy and confirmatory biopsy, and continued in AS, of which totaled 173 patients.

Between September 2006 – August 2017, 89 patients were enrolled into the UKM's PRIAS cohort. We queried all available AS medical record data from these patients. We then selected for all patients who underwent diagnostic biopsy and confirmatory biopsy, and continued in AS, of which totaled 58 patients.

**Table 4.** Cohort Demographics of the 380 patients in Study I. Modified from the submitted manuscript "Cumulative Cancer Locations is a Novel Metric to Predict Active Surveillance Outcomes: A Multi-Center study".

| Characteristics | HUS (n= 149) | IEO (n= 173) | UKM (n= 58) | Pooled (n= 380) |
|---|---|---|---|---|
| Age at Diagnosis, years, median (range) | 63.19 (41 - 78) | 64.57 (44 - 78) | 65.31 (50 - 75) | 63.1 (41 - 78) |
| Pre-AS PSA, ng/ml, median (range) | 5.5 (0.9 - 10) | 5.61 (0 - 50.7) | 6.05 (1.7 - 16.7) | 5.68 (0 - 50.7) |
| PSA-D, median (range) | 0.14 (0.05-0.2) | 0.10 (0.02 - 0.41) | 0.14 (0.04 - 0.31) | 0.12 (0.02 - 0.41) |
| pT Stage at Dg (n, %) | | | | |
| T1c | 148 (99.3) | 159 (91.9) | 48 (82.8) | 355 (93.4) |
| T2a | 1 (0.7) | 14 (8.1) | 10 (17.2) | 25 (6.6) |
| Number of DgBx positive cores (n, %) | | | | |
| 1 | 116 (77.9) | 118 (68.2) | 40 (69.0) | 274 (72.1) |
| 2 | 33 (22.1) | 48 (27.2) | 18 (31.0) | 99 (26.1) |
| 3* | NA | 7 (4.0) | NA | 7 (1.8) |
| Number of CBx positive cores (n, %) | | | | |
| 0 | 74 (59.8) | 93 (53.8) | 35 (60.3) | 201 (56.9) |
| 1 | 32 (26.2) | 38 (22.0) | 20 (34.5) | 90 (25.5) |
| 2 | 17 (13.9) | 29 (16.8) | 3 (5.2) | 49 (13.9) |
| 3* | NA | 13 (7.5) | NA | 13 (3.7) |
| Discontinuation (n, %) | | | | |
| Protocol Based | 34 (59.6) | 37 (56.9) | 18 (78.2) | 89 (61.4) |
| Gleason Upgrade** | 18 (52.9) | 12 (32.4) | 13 (56.5) | 43 (48.3) |
| Non-Protocol Based | 23 (43.4) | 28 (43.1) | 5 (21.7) | 56 (38.6) |
| Active Treatment (n, %) | | | | |
| RP | 30 (52.6) | 59 (85.5) | 12 (52.2) | 101 (67.8) |
| RT | 8 (14.0) | 10 (14.5) | 7 (30.4) | 25 (16.8) |
| WW / Other | 19 (33.3) | 0 (0.0) | 4 (17.4) | 23 (15.4) |

RP, radical prostatectomy; RT, Radiotherapy; WW, Watchful-Waiting; PSA, prostate-specific antigen; PSA-D, prostate-specific antigen density; pT, pathological tumor stage. *IEO AS criteria allow for 3 positive cores. **Gleason-Upgrade percentage assessed as proportion of Protocol-Based Discontinuers.

## Study II:

For Study II, the study cohort comprised 231 patients enrolled in the HUS PRIAS trial between October 2006 and March 2013. Of these patients, 203 had archival pathology blocks available for research use. Detailed Study II cohort demographics can be viewed in Table 5.

**Table 5 -** Cohort Demographics of the 203 patients in Study II. Modified from the table published in "PTEN Loss but Not ERG Expression in Diagnostic Biopsies Is Associated with Increased Risk of Progression and Adverse Surgical Findings in Men with Prostate Cancer on Active Surveillance". Lokman et al., in Eur Urol Focus, reprinted by permission of Elsevier on behalf of Eur Urol Focus.

| | Study population (n=203) | PTEN positive (n=161) | PTEN negative (n = 29) |
|---|---|---|---|
| Age at diagnosis, years, median (IQR) | 63.4 (59.4 - 68.0) | 63.4 (59.4 - 67.9) | 63.4 (59.4 - 69.3) |
| PSA, ng/ml, median (IQR) | 5.6 (4.4 - 6.9) | 5.6 (4.35 - 6.7) | 5.9 (4.2 - 7.8) |
| PSA density, median (IQR) | 0.14 (0.11 - 0.16) | 0.14 (0.11 - 0.16) | 0.13 (0.11 - 0.16) |
| Prostate volume, median (IQR) | 40.4 (33.0 - 50.3) | 40.4 (33.5 - 50.5) | 43.4 (35.3 - 53.0) |

| | | | |
|---|---|---|---|
| Number of benign biopsies prior to diagnosis, n (%) | | | |
| 0 | 147 (72.4) | 114 (70.8) | 24 (82.8) |
| 1 | 42 (20.7) | 35 (21.7) | 4 (13.8) |
| 2 | 10 (4.9) | 8 (5.0) | 1 (3.4) |
| 3 | 3 (1.5) | 3 (1.9) | 0 (0) |
| 4 | 1 (0.5) | 1 (0.6) | 0 (0) |
| Total number of PRIAS biopsy sessions[#] (n = 180), n, (%) | | | |
| 1 | 14 (7.3) | 12 (7.8) | 1 (3.7) |
| 2 | 69 (35.8) | 53 (34.6) | 13 (48.1) |
| 3 | 57 (29.5) | 47 (30.7) | 5 (18.5) |
| 4 | 35 (18.1) | 30 (19.6) | 3 (11.1) |
| 5 | 13 (6.7) | 6 (3.9) | 5 (18.5) |
| 6 | 5 (2.6) | 5 (3.3) | 0 (0) |
| Number of positive cores at diagnosis (n = 203), n, (%) | | | |
| 1 | 150 (73.9) | 119 (73.9) | 19 (65.5) |
| 2 | 53 (26.1) | 42 (26.1) | 10 (34.5) |
| Cancer location (n = 194), n, (%) | | | |
| Unilateral | 175 (90.2) | 139 (89.7) | 25 (89.3) |
| Bilateral | 19 (9.8) | 16 (10.3) | 3 (10.7) |
| Biopsy ERG staining (n = 190) | | | |
| Positive | 74 (38.9) | 56 (35.2) | 17 (58.6) |
| Negative | 116 (61.1) | 103 (64.8) | 12 (41.4) |
| PSA-DT ≤ 3 years at 1 year follow-up (n = 203) | 155 (76.4) | 122 (75.8) | 21 (72.4) |
| Age at discontinuation, median (IQR) | 66.3 (61.8 - 70.5) | 64.82 (61.1-67.3) | 66.7 (61.9 - 71.0) |
| PRIAS status (n = 203) | | | |
| Non-protocol based discontinuation* | 34 (16.7) | 27 (16.8) | 5 (17.2) |
| Protocol based discontinuation | 72 (35.5) | 53 (32.9) | 15 (51.7) |
| Continuing on PRIAS | 97 (47.8) | 81 (50.3) | 9 (31.0) |

- * 4 discontinued due to anxiety, 21 discontinued to watchful waiting, 5 died for other reasons, and 4 discontinued due MRI findings
- [#] 1 = diagnostic biopsy, 2-6 re-biopsies

Study III:

For Study III, the study cohort comprised 831 patients who were treated with radical prostatectomy, of whom 358 were treated in Helsinki between 1983 and 1998, and of whom 457 were treated in Turku between 2000 and 2005. Of these patients, 815 had representative tissue-microarray material available for analysis. Detailed Study III cohort demographics can be viewed in Table 6.

**Table 6. -** Cohort Demographics of the 815 patients in Study III. Reprinted with permission from Study III.

| | *Helsinki cohort (1983-1998) (n=358)* | *Turku cohort (2000-2005) (n=457)* | *Total (n=815)* |
|---|---|---|---|
| Age at RP, years (mean, SD) (*n* = 815) | 63.4 (5.9) | 61.6 (5.8) | 62.4 (5.9) |
| Preoperative PSA, ng/ml (n, %) (*n* = 708) | | | |
| ≤10.0 | 143 (50.5) | 294 (69.2) | 437 (61.7) |
| 10.1-20.0 | 89 (31.4) | 96 (22.6) | 185 (26.1) |
| >20.0 | 51 (18.0) | 35 (8.2) | 86 (12.2) |
| Gleason score at RP (n, %) (*n* = 815) | | | |
| ≤6 | 93 (26.0) | 168 (36.8) | 261 (32.0) |
| 7 | 207 (57.8) | 197 (43.1) | 404 (49.6) |
| 8-10 | 58 (16.2) | 92 (20.1) | 150 (18.4) |
| Grade group at RP (n, %) (*n* = 815) | | | |
| 1 | 93 (26.0) | 168 (36.8) | 261 (32.0) |
| 2 | 93 (26.0) | 134 (29.3) | 227 (27.9) |
| 3 | 114 (31.8) | 63 (13.8) | 177 (21.7) |
| 4 | 45 (12.6) | 70 (15.3) | 115 (14.1) |
| 5 | 13 (3.6) | 22 (4.8) | 35 (4.3) |
| pT (n, %) (*n* = 774) | | | |
| 2 | 202 (60.5) | 233 (53.0) | 435 (56.2) |
| 3 | 122 (39.5) | 207 (47.0) | 339 (43.8) |
| Lymph node status (n, %) (*n* = 806) | | | |
| Negative | 342 (97.2) | 434 (95.6) | 776 (96.3) |
| Positive | 10 (2.8) | 20 (4.4) | 30 (3.7) |

| | | | |
|---|---|---|---|
| ERG status in TMA (n, %) (*n* = 815) | | | |
| Any core positive | 181 (50.6) | 228 (49.9) | 406 (49.8) |
| Negative | 177 (49.6) | 229 (50.1) | 409 (50.2) |
| PTEN status in TMA (n, %) (*n* = 815) | | | |
| Intact | 164 (45.8) | 338 (74.0) | 502 (61.6) |
| Any loss | 194 (54.2) | 119 (26.0) | 313 (38.4) |
| Complete loss | 77 (21.5) | 58 (12.7) | 135 (16.6) |
| AR status in TMA (n, %) (*n* = 358) | | | |
| Low | 127 (35.5) | n.a. | |
| High | 231 (64.5) | n.a. | |
| Follow-up time after RP, years (median, range) (n=815) | 15.7 (0.7-28.6) | 9.5 (0.2-14.0) | 11.9 (0.2-28.6) |
| Death from any cause (n, %) (*n* = 815) | 172 (48.0) | 73 (16.0) | 245 (30.0) |
| Death from prostate cancer (n, %) (*n* = 815) | 33 (9.2) | 19 (4.2) | 52 (6.4) |
| Patients receiving secondary therapy after RP (n, %) (*n* = 796) | 124 (34.6) | 136 (31.1) | 260 (32.7) |

Study IV:

For Study IV, the study cohort comprised n = 831 patients who were treated with radical prostatectomy, of whom n = 358 were treated in Helsinki between 1983 and 1998, and of whom n = 457 were treated in Turku between 2000 and 2005. Detailed Study III cohort demographics can be viewed in Table 7.

**Table 7. Cohort Demographics of the 831 patients in Study IV. Modified from the table published in "New prostate cancer grade grouping system predicts survival after radical prostatectomy". Erickson et al., in Hum Path. reprinted by permission of ¬__ on behalf of Hum Path.**

| Characteristics | Helsinki cohort (1982-1998) | Turku cohort (2000-2005) | Total |
|---|---|---|---|
| Age at RP, years (n = 831) (median, range) | 64 (45-76) | 62 (40-73) | 63 (40 - 76) |
| Diagnostic PSA, ng/ml (n = 715) (n, %) | | | |
| ≤10.0 | 145 (50.0) | 294 (69.2) | 439 (61.4) |
| 10.1-20.0 | 92 (31.7) | 96 (22.6) | 188 (26.3) |
| >20.0 | 53 (18.3) | 35 (8.2) | 88 (12.3) |
| Gleason score at RP (n = 831) (n, %) | | | |
| ≤6 | 97 (25.9) | 168 (36.8) | 265 (31.9) |
| 7 | 217 (58.0) | 197 (43.1) | 414 (49.8) |
| 8-10 | 60 (16.0) | 92 (20.1) | 152 (18.3) |
| Grade Group at RP (n = 831) (n, %) | | | |
| 1 | 97 (25.9) | 168 (36.8) | 265 (31.9) |
| 2 | 95 (25.4) | 134 (29.3) | 229 (27.6) |
| 3 | 122 (32.6) | 63 (13.8) | 185 (22.3) |
| 4 | 47 (12.6) | 70 (15.3) | 117 (14.1) |
| 5 | 13 (3.5) | 22 (4.8) | 35 (4.2) |
| pT (n = 784) (n, %) | | | |
| 2 | 208 (60.5) | 233 (53.0) | 441 (56.3) |
| 3 | 136 (39.5) | 206 (46.8) | 343 (43.8) |
| Lymph node status (n = 822) (n, %) | | | |
| N0 | 358 (97.3) | 434 (95.6) | 792 (96.4) |
| N1 | 10 (2.7) | 20 (4.4) | 30 (3.6) |
| Follow-up time after RP, years (n = 831) (median, range) | 15.7 (0.1-28.6) | 9.5 (0.2-14.0) | 11.1 (0.1-28.6) |
| Death from any cause (n = 831) (n, %) | 183 (48.9) | 73 (16.0) | 256 (30.8) |
| Death from prostate cancer (n = 831) (n, %) | 36 (9.6) | 19 (4.2) | 55 (6.6) |
| Patients receiving secondary therapy (n = 812) (n, %) | 128 (34.2) | 136 (31.1) | 264 (31.8) |

**Abbreviations: RP indicates radical prostatectomy; PSA, prostate-specific antigen; pT, pathological stage**

RP, radical prostatectomy; PSA, prostate-specific antigen; pT, pathological tumor stage; TMA, tissue microarray; AR, androgen receptor

# 4.2 Cumulative Number of Cancer Locations (I)

Study I analyzed a novel metric, named cumulative number of cancer locations (CCLO), in predicting AS outcomes. Cumulative cancer locations (CCLO) is calculated by determining sextant positive locations in an individual bi-sextant Bx session (Figure 17A) and summing the total distinct sextant locations from both diagnostic biopsy and confirmatory biopsy (Figure 17B).

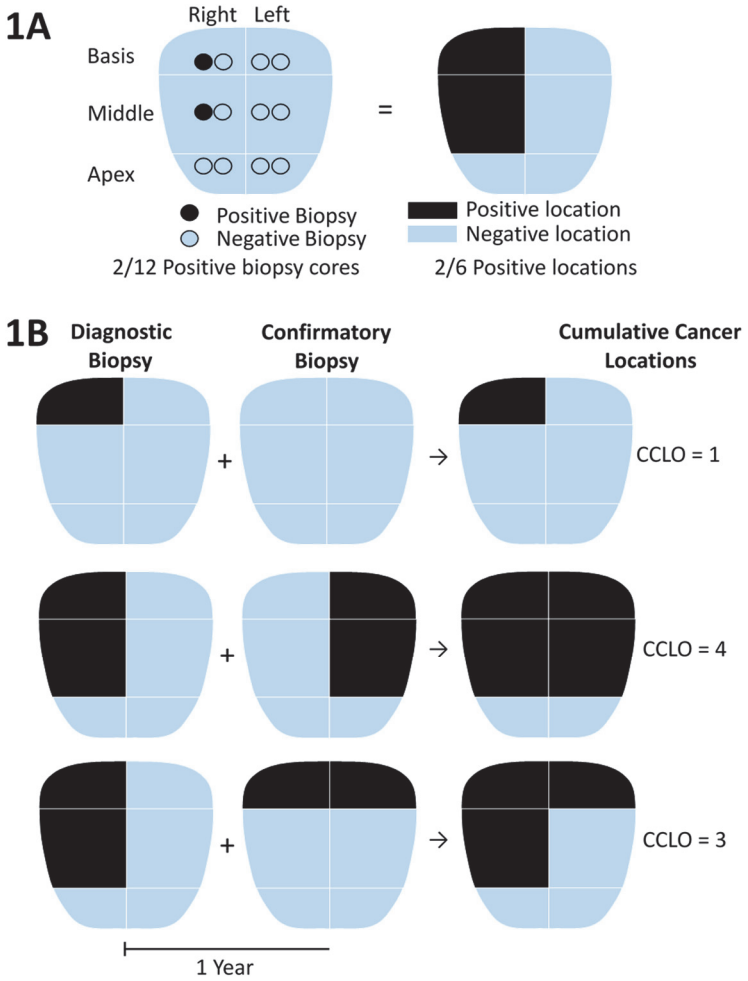


*Figure 17. Calculation of CCLO. Modified from Figure submitted in Study I.*

## 4.3 Tissue samples and microarrays (II & III)

Finnish Act on the Medical Use of Human Organs, Tissues and Cells (No. 101/2001), lays out the permission for secondary use of patient tissues for medical research, which were utilized in studies II & III.

Patient tissue materials were obtained in accordance with ethical approvals as further described in section 4.9.

Tissue micro arrays (TMAs) is a method of tissue processing allowing for the ability to study many patient's materials in parallel in a cost and time efficient manner (Kallioniemi *et al*, 2001). Briefly, FFPE tissues are transferred by 'punching', wherein a cylindrical core of tissue is transferred from a donor block to a recipient block. The recipient blocks can contain hundreds of patients' samples, depending on the selected core size. The prepared TMA blocks can then sectioned, and the research methodologies can be performed on the TMA slides. The TMA concept is presented in Figure 18.

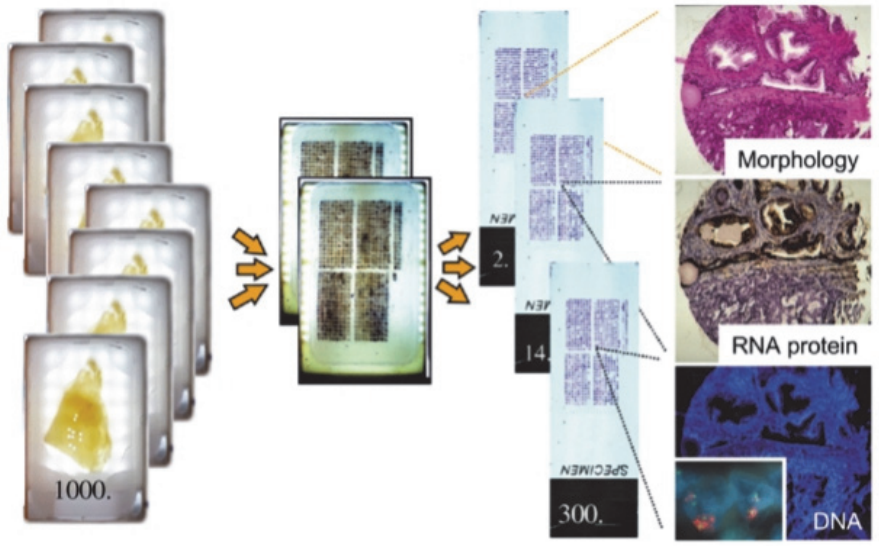


*Figure 18. Tissue micro array method. Reproduced with permission from* (Kallioniemi *et al*, 2001) *(Permission obtained 29.1.2018)*

Study II utilized HUS PRIAS patient's diagnostic biopsy and radical prostatectomy tissue microarrays derived from radical prostatectomy sections for patients who underwent surgery. Patient's diagnostic biopsy and radical prostatectomy data (for those who underwent radical prostatectomy) were queried from Qpati (laboratory information management system developed by Tieto), and blocks and slides were identified, and retrieved from HUS Pathology archives (Figure 19). The slides were then reviewed, and diagnostic biopsy containing cancer were selected for further sectioning. Radical prostatectomy slides were reviewed, marked, and tissue microarrays were constructed using two, 1 mm thick, TMA cores per marked area. Sections were cut from diagnostic

biopsy and radical prostatectomy tissue-microarray FFPE blocks, 0,4 uM thick, mounted on slides.

Study III utilized tissue microarrays from patients who underwent radical prostatectomy in Helsinki and Turku. Data were queried as described previously, slides retrieved, and three cancer areas, and one benign area, per patients' radical prostatectomy, were punched into TMA format. Sections were cut from TMA FFPE blocks, 0,4 µM thick, mounted on slides.

*Figure 19.* HUS Department of Pathology archives containing histological slides, and formalin fixed-paraffin embedded (FFPE) blocks taken during clinical care of HUS patients. (Image printed with permission of HUS Pathology, obtained 17.01.2018. Original photo taken by the author 01.04.2016).

## 4.4 Immunohistochemistry (II & III)

For Studies II and III, IHC stainings were performed using an autostainer (Dako A/S, Glostrup, Denmark) as described in their respective publications per manufacturer guidelines. Briefly, immunohistochemistry was performed after heat-induced epitope retrieval on consecutive slides

with 1:100 dilution for PTEN antibody (D4.3 XP, Cell Signaling Technology, Danvers, MA, USA) and with a 1:300 dilution for ERG antibody (EPR 3864, Abcam PLC, Cambridge, UK). Please see Table 8 for further details about antibodies and IHC performed in studies II & III.

Table 8. Primary antibodies

| Antigen | Supplier | Product # | Dilution | HIER Buffer | Study |
|---|---|---|---|---|---|
| AR | | | 1:750 | TRIS-EDTA pH 9.0 | III |
| ERG | Abcam | EPR 3864 | 1:300 | Citrate pH 6.0 | II & III |
| PTEN | CST | 9188 | 1:100 | TRIS-EDTA pH 9.0 | II & III |

# 4.5 Slide Scanning (II & III)

Digital whole-slide images were acquired at 0.26 µm/pixel resolution using a Pannoramic P250 Flash II whole slide scanner (3DHistech, Hungary) equipped with a Zeiss Plan-Apochromat 20x objective (NA 0.8). Images were converted to the JPEG2000 format (95% quality) and uploaded to the WebMicroscope virtual microscope platform (Fimmic, Finland) for visual inspection (Lundin *et al*, 2004).

# 4.6 Slide Scoring

The digitized slides were then scored independently by observers: in Study II, an experienced board-certified pathologist and trained urological resident, and in Study III, three board-certified pathologists. Cytoplasmic PTEN expression was scored as either “PTEN Intact” (Figure 20B) or “PTEN Loss” (Figure 20D, 21A, 21D) for both studies II & III. Nuclear ERG expression was scored as either “ERG Positive” (Figure 20A, 21B) or “ERG Negative” (Figure 20C, 21E). Nuclear AR expression was scored in a dichotomous manner as either “AR High” (Figure 21C, 21F) or “AR Low (Not shown).

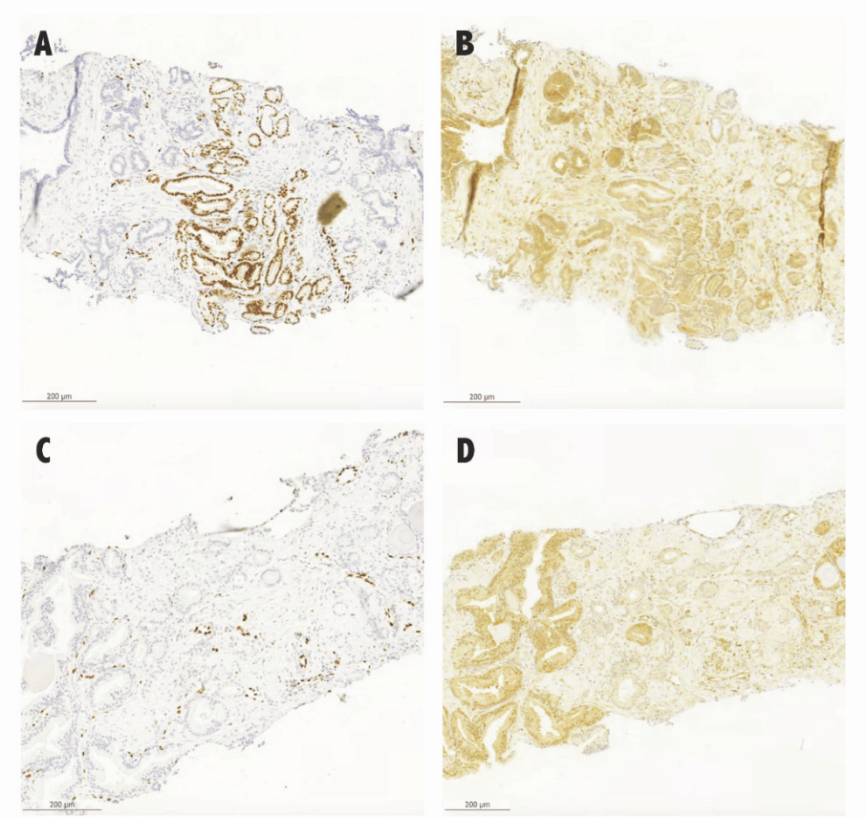


*Figure 20. Representative images of ERG and PTEN immunohistochemical stainings in diagnostic biopsies of patients who underwent active surveillance. A) ERG positive diagnostic biopsy, B) PTEN Intact diagnostic biopsy, C) ERG Negative diagnostic biopsy, D) PTEN loss diagnostic biopsy. (Printed with permission from Study II)*

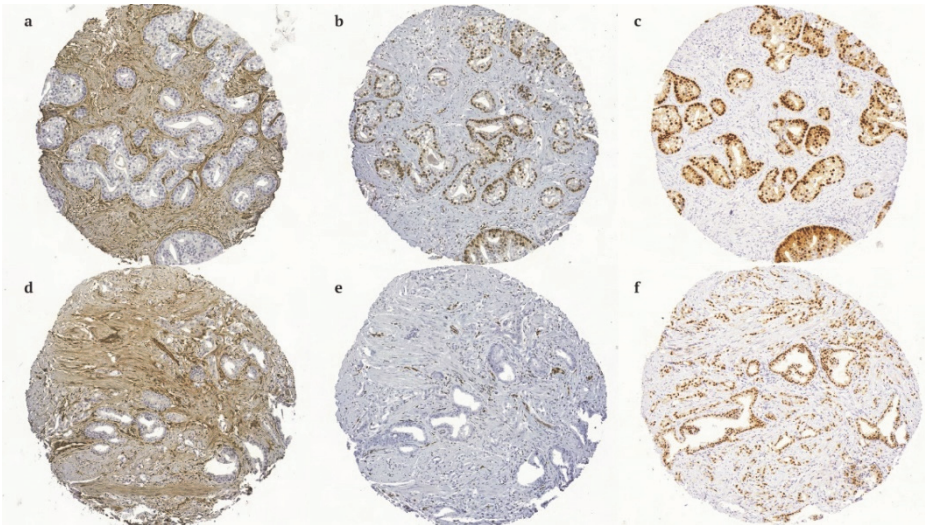


*Figure 21.* Representative images of PTEN, ERG, and AR immunohistochemical stainings in tissue microarray spots of patients who underwent radical prostatectomy (Study III). A) PTEN Loss, B) ERG Positive, C) AR Strongly Positive, D) PTEN Loss, E) ERG Negative, F) AR Strongly Positive. (Reprinted with permission from Study III, 20.01.2018)

## 4.7 Statistics (I-IV)

### 4.7.1 Study 1

Student's t-tests and Fisher's exact tests were performed to assess study outcomes and standard clinical variables. Mantel-Haenszel log-rank tests were used to compare Kaplan-Meier curves. Such Kaplan-Meier curves were plotted for total CCLO substrata from each individual Study I cohort individually and the pooled cohort by time to event for each study outcome. Kaplan-Meier curves were also plotted for nested subcohorts of the pooled cohorts, for patients from all three centers, with 0 and 1 positive confirmatory biopsy, sub-stratified by total CCLO, and analyzed with respect to all three study outcomes. Additionally, Kaplan-Meier curves were plotted for the pooled study cohort, substratified by 3-tier CCLO, and analyzed with respect to time until the three study outcomes. Multivariate cox regression analyses of positive cores in diagnostic biopsy and confirmatory biopsy, total CCLO, and 3-tier CCLO, were analyzed independently with Age at Dg, PSA, PSA-D, cTstage. Receiver operating characteristic (ROC) curves were plotted and area under the curves (AUC) were calculated for the sensitivity and specificity of both confirmatory biopsy and 3-tier CCLO to predict all three study outcomes. Decision curves were plotted and analyzed (DCA) for to compare

confirmatory biopsy and 3-tier CCLO with regards to their ability to predict outcomes for individual patients across different risk-of-event thresholds. All p-values were adjusted by the Benjamini-Hochberg method. All prior mentioned analyses were performed in R version 3.3.2, using the packages *survminer* (Kassambara & Kosinski, 2017), *survival* (Therneau & Grambsch, 2000), *rms* (Harrell, 2015), *pROC* (Harrell, 2015), and *dca* (Harrell, 2015).

### 4.7.2 Study 2

Study II utilized the same AS outcomes as Study I; Gleason upgrade, Protocol-Based Discontinuation, and adverse radical prostatectomy findings. Univariate and multi-variate Cox regression analyses were performed to assess marker status, age, PSA, diagnostic biopsy core positivity, and number of biopsy session with regards to study outcomes. Kaplan-Meier curves were compared by Mantel–Haenszel log rank statistics. As discontinuing due to other Protocol-Based AS criteria is a competing outcome of Gleason Upgrade, we performed Gray's competing risk analysis (Fine & Gray, 1999) to determine whether there were differences in survival profiles by biomarker status strata. In order to analyze the distribution of biomarker status in radical prostatectomies as compared to Bx, chi-squared analyses were performed. All statistical analyses were performed in R v3.3.2, or IBM SPSS v23.

### 4.7.3 Study 3

Study III utilized the primary study outcomes of Disease-specific survival and Overall-survival, as well as a secondary outcome of initiation of secondary therapies. Fisher's exact tests, as well as chi-squared analyses, were utilized to analyze the association between clinical variables and biomarker status. Mantel-Cox test were used to compare Kaplan-Meier plots for biomarker status and outcomes. Univariate and multivariate Cox regression analyses were utilized to compare age at operation, PSA, pT stage, Gleason score, and lymph node status, with biomarker status with respect to study outcomes. IBM SPSS Statistics version 23 was used for all statistical analyses.

### 4.7.4 Study 4

Study IV utilized prostate cancer-specific mortality and all-cause mortality as primary study outcomes, and in the Helsinki cohort alone, initiation of

secondary therapy was a secondary study outcome. Follow-up was limited to 15 years, and if outcome events occurred after 15 year follow-up, the outcome was censored and follow-up encoded as 15 years. Kaplan-Meier plots were performed to compare GG and all study outcomes, and were compared using the Mantel-Haenzel log-rank method. Cox regression (uni and multivariate) analyses were performed to compare age GG, in addition to age at operation, PSA at diagnosis, pathological T-stage (pT), and lymph node status with respect to study outcomes. As any-cause mortality is a competing risk for prostate-cancer specific mortality, we performed Gray's competing risk analysis to compare whether there was a difference in survival profile if substratifying the cohort by Grade group. Receiver operating characteristic (ROC) curves were plotted, and area under the curve (AUC) was analyzed to compare the 3-tier Gleason score system with the 5-tier Grade Group system for sensitivity and specificity to predict outcomes. Decision curves were ploted, and analyses (DCA) were performed to compare GG and GS with respect to study outcomes. All statistical analyses for study IV were performed using R Statistical Software v.3.3.3, using the *rms*, *pROC*, *survminer*, *survival*, *cmprsk*, and *dca* packages.

## 4.8 Ethical Approvals (I-IV)

Studies I was performed using data from HUS and UKM arms of the prospective PRIAS trial (HUS 276/E6/06), where patients gave prospective informed consent. Patients in the IEO cohort of study I also gave prospective consent before enrolling in IEO active surveillance. Study II utilized data from the HUS PRIAS trial, as described previously, however, the study also utilized patient tissue material approved by HUS (214/2016) and THL (490/5.05.00/2016). Data for studies III and IV were obtained retrospectively from primary patient records, and registry data such that informed consent was not required.

# 5. Results

## 5.1 Study 1

Study I cohorts were selected as described in Section 4.1. We first analyzed clinicopathological variables and their association with study outcomes using t-tests and fisher's exact tests, which showed that CCLO was associated with outcomes (please see Original Publication I).

After establishing that CCLO was associated with study outcomes, we then sought to determine whether there was a difference in the distribution of positive cores and CCLO during the diagnostic phase of AS. In univariate chi-squared analyses, there was a difference in distribution of diagnostic biopsy positive cores and locations ($P < 0.001$), confirmatory biopsy positive cores and CCLO ($P < 0.001$), and diagnostic biopsy and Confirmatory biopsy positive cores and CCLO ($P < 0.001$) (results not shown). These results suggest that there is a difference in distribution of positive cores and CCLO.

We then thought, that while there may be an empirical difference in the distribution, we asked whether or not this difference was clinically relevant. In order to answer this question, we conducted Kaplan-Meier analyses where we subgrouped the pooled cohort by the number of confirmatory biopsy positive cores, and substratified these groups by CCLO. Patients with higher CCLO within those with one positive confirmatory biopsy were significantly more likely to experience shorter protocol-based discontinuation and Gleason upgrade-free survival than those with lower CCLO (Figure 22 B1-B2, $P < 0.008$). Similar results were found for patients with zero positive confirmatory biopsy, please see Original Publication I. We attempted to do similar analyses on patient groups with two and higher confirmatory biopsy, however, the number of patients in the subgroups and number of outcome events became too small to meaningfully analyze.

Having established there may be a difference in outcomes between positive cores and CCLO, we then conducted Kaplan-Meier analyses in individual subcohorts separately (HUS, IEO, and UKM), and the pooled cohort for all three study outcomes to determine whether or not there was a difference in survival profiles based on CCLO. Higher CCLO was associated with worse study outcomes in all three cohorts ($P < 0.05$, please see Original Publication I). Furthermore, in pooled analysis of all three study cohorts, higher CCLO was associated with protocol-based

discontinuation, Gleason upgrade, and adverse radical prostatectomy findings (P < 0.001, Figure 23 D1-D3). Intriguingly, there seemed to be a dose-dependent effect of CCLO on worse outcomes.

In order to assess whether CCLO was independently associated with outcomes, we conducted multivariate Cox regression analysis containing other standard AS clinicopathological variables. As CCLO is partially calculated by confirmatory biopsy, collinearity becomes an issue when trying to analyze both in the same regression model, so we thus ran separate models with the same variables: one containing all variables + diagnostic biopsy and confirmatory biopsy, and another containing all variables + CCLO. In multivariate cox regression analysis, higher CCLO was a significant independent predictor of increased risk for poor AS outcome (P < 0.05, please see Original Publication I).

Given the prior results, we then considered how the results may be considered in a clinical context. Treating physicians risk-stratify patients into low, intermediate, and high-risk groups, and make clinical decisions based on these risk-profiles. Our results suggested that patients with one CCLO formed a “low-risk” group, patients with two CCLO formed a “intermediate-risk” group, and patients with three or higher CCLO formed a “high-risk” group.

To assess this 3-tier CCLO risk grouping, we conducted Kaplan-Meier analyses of risk groups by study outcomes in the pooled cohort. Higher CCLO risk group patients were significantly more likely to experience Gleason upgrade, protocol-based discontinuation, and adverse radical prostatectomy findings (p < 0.002, Figure 24 2A-C) as compared to intermediate and low-risk CCLO groups.

In order to further assess the independent predictive ability of the 3-tier CCLO risk-stratification to predict outcomes, we conducted multivariate Cox regression analysis using the same clinicopathological AS variables as described previously. Patients in the high risk CCLO group were significantly more likely to experience Gleason upgrade, protocol-based discontinuation, and adverse radical prostatectomy findings as compared to those in the low risk CCLO groups (Table 9, P < 0.007). These results strongly suggested that indeed the 3-tier CCLO risk stratification can predict AS outcomes.

Given our original study aim, we then sought to determine how the 3-tier CCLO risk stratification may be used in clinical practice. Given that individual bx results are considered at static time points, we sought to

compare the current clinical standard, Confirmatory biopsy positive cores to the 3-tier CCLO risk stratification. In receiver-operating characteristic (ROC) area under the curve (AUC) analyses, 3-tier CCLO risk grouping outperformed confirmatory biopsy in predicting protocol-based discontinuation (0.734 vs 0.682), Gleason upgrade (0.655 vs 0.576) and adverse radical prostatectomy findings (0.662 vs 0.561) (please see Original Publication I for figures). These results suggest that the 3-tier CCLO risk stratification outperforms confirmatory biopsy.

While ROC-AUC analysis is useful in quantifying and comparing the difference between prognostic predictors, it can be difficult to apply to individual patients, as patients have different risk-profiles with regards to outcomes. To address this, we then employed decision curve analyses (DCA) to compare confirmatory biopsy and 3-tier CCLO with regards to all three study outcomes. DCA, though not well known by many clinicians, is designed to assess the difference between different predictors at different risk-probabilities of outcomes. The y-axis is “net benefit”, which is a quantification of the benefit of making a decision. On the x-axis, “threshold probability” is plotted, which is representative of the probability of the measured outcome event occurring from 0-100%. Two lines are always plotted in DCA, the first being a horizontal line of 0.0 net benefit across all threshold probabilities, representing if no patients were treated. A second line is plotted representing if all patients were treated. Ideally, predictors are plotted in positive net benefit regions above the line if all patients were treated. The analysis is designed to allow a treating physician to utilize their clinical knowledge of the risk profile of the individual patient for a specific outcome, and then evaluate which prognostic test is best to use for that specific patient. In decision curve analysis, 3-tier CCLO outperformed confirmatory biopsy at almost all observed threshold probabilities of all three study outcomes (Figure 25 3B1-3). These results give further evidence to the increased ability of the 3-tier CCLO risk stratification to predict poor AS outcomes as compared to confirmatory biopsy.

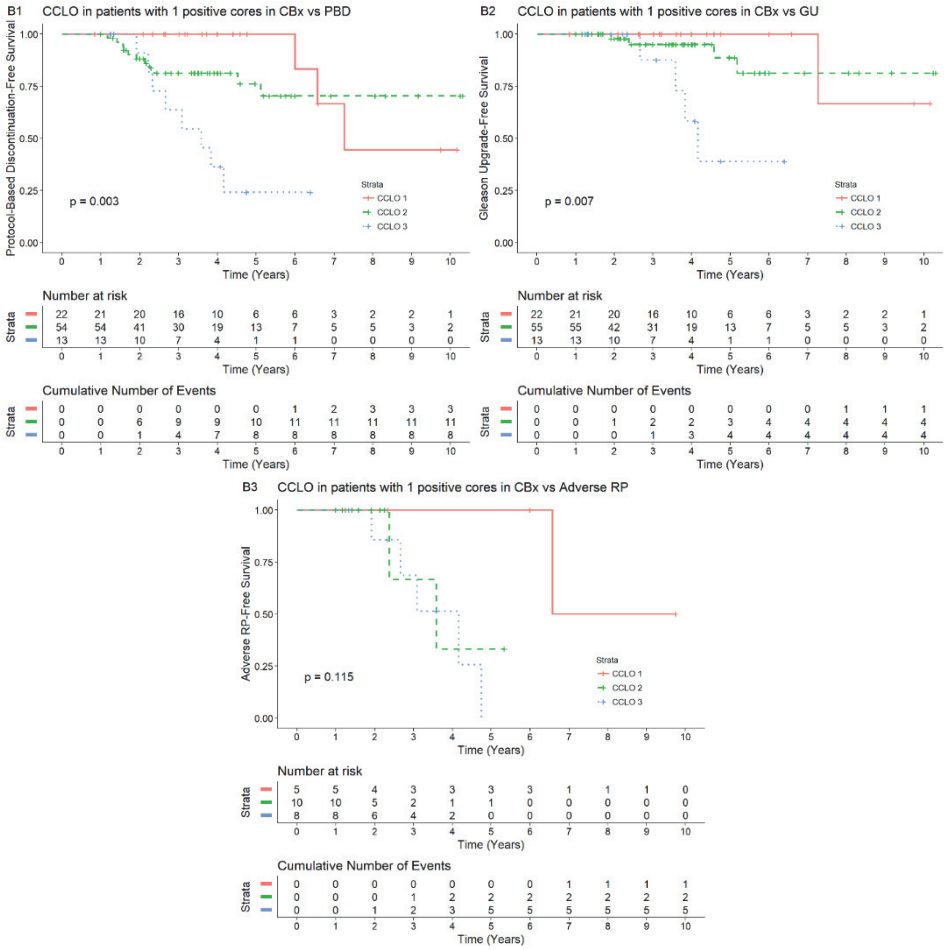


*Figure 22.* Kaplan-Meier curves of patients with 1 positive core in confirmatory biopsy (CBx), as stratified by the cumulative number of cancer locations (CCLO) with respect to B1) Protocol-Based Discontinuation (PBD), B2) Gleason-Upgrade Based Discontinuation (GU), and adverse findings in RP specimens (Adverse RP). *Reprinted with permission from Study I.*

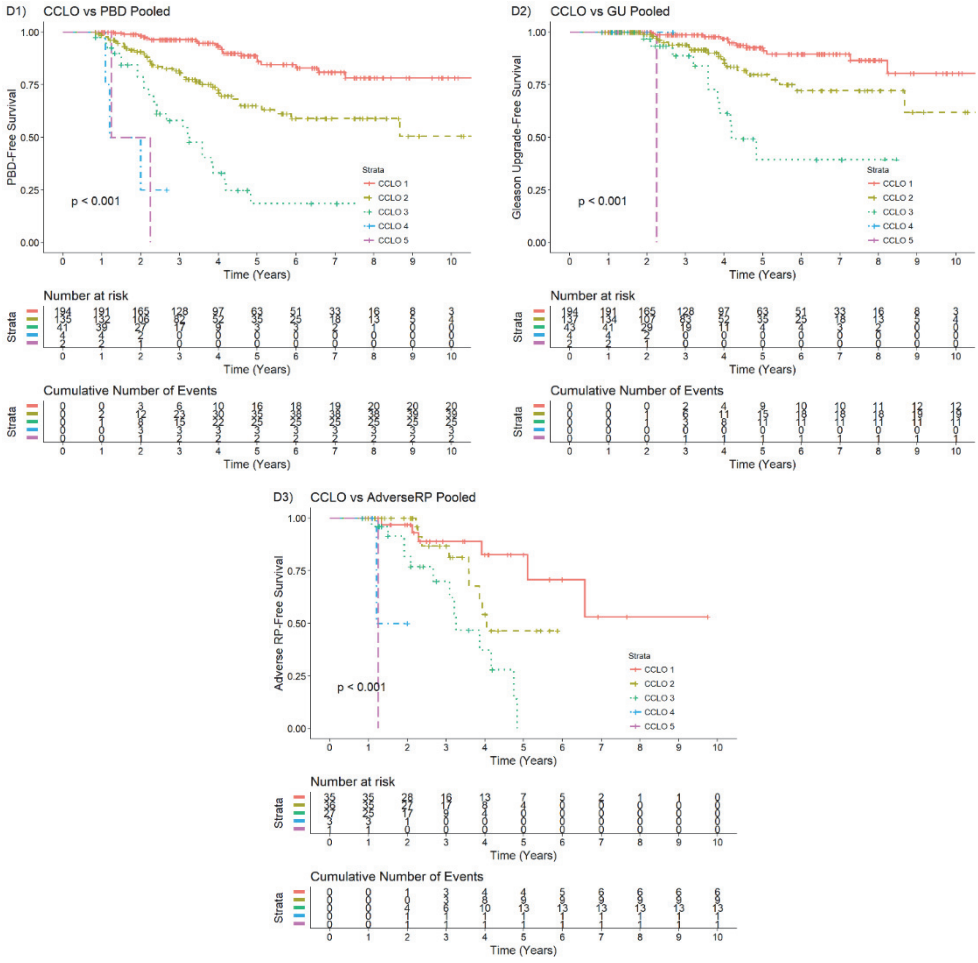


*Figure 23.* Kaplan-Meier curves of cumulative number of cancer locations (CCLO) for D1) Protocol-based discontinuation, D2) Gleason-Upgrade based Discontinuation (GU), D3) Adverse findings in RP specimens (Adverse RP) in the pooled study cohort. Reprinted with permission from Study I.

**Table 9A**. Multivariate cox regression analysis of 3-tier CCLO and number of positive cores in CBx vs protocol-based discontinuation. Pooled analysis of all three AS cohorts. Reprinted with permission from Study I.

| | HR | 95% CI | P-Value |
|---|---|---|---|
| Age At Dg | 0.96 | (0.92 - 0.99) | **0.035** |
| PSA | 0.96 | (0.86 - 1.08) | 0.6 |
| PSA-D[a] | 1.77 | (1.26 - 2.50) | **0.004** |
| Clinical Tstage 1 | - | - | Reference |
| Clinical Tstage 2 | 3.47 | (1.74 - 6.93) | **0.002** |
| CCLO Low Risk | - | - | Reference |
| CCLO Intermediate Risk | 3.31 | (1.81 - 6.07) | **0.001** |
| CCLO High Risk | 12.15 | (6.18 - 23.9) | **<0.001** |

[a] HR for PSA-D is reported per 0.10 increase.
CCLO = Cumulative Number of Cancer Locations; CBx = Follow-up Biopsy; Dg = Diagnosis; PSA = Prostate-specific antigen; PSA-D = Prostate-specific antigen density

**Table 9B**. Multivariate cox regression analysis of 3-tier CCLO and number of positive cores in CBx vs Gleason Upgrade Based discontinuation. Pooled analysis of all three AS cohorts.

| | HR | 95% CI | P-Value |
|---|---|---|---|
| Age At Dg | 0.98 | (0.93 - 1.03) | 0.5 |
| PSA | 0.92 | (0.78 - 1.09) | 0.5 |
| PSA-D[a] | 1.93 | (1.13 - 3.31) | **0.035** |
| Clinical Tstage 1 | - | - | Reference |
| Clinical Tstage 2 | 3.88 | (1.29 - 11.7) | **0.035** |
| CCLO Low Risk | - | - | Reference |
| CCLO Intermediate Risk | 2.58 | (1.19 - 5.61) | **0.035** |
| CCLO High Risk | 6.01 | (2.16 - 16.8) | **0.002** |

[a] HR for PSA-D is reported per 0.10 increase.
CCLO = Cumulative Number of Cancer Locations; CBx = Follow-up Biopsy; Dg = Diagnosis; PSA = Prostate-specific antigen; PSA-D = Prostate-specific antigen density

**Table 9C**. Multivariate cox regression analysis of 3-tier CCLO and number of positive cores in CBx vs Adverse RP Findings. Pooled analysis of all three AS cohorts.

| | HR | 95% CI | P-Value |
|---|---|---|---|
| Age At Dg | 1.02 | (0.93 - 1.12) | 0.8 |
| PSA | 0.99 | (0.80 - 1.23) | 1.0 |
| PSA-D[a] | 1.11 | (0.59 - 2.09) | 0.8 |
| Clinical Tstage 1 | - | - | Reference |
| Clinical Tstage 2 | 1.45 | (0.25 - 8.37) | 0.8 |
| CCLO Low Risk | - | - | Reference |
| CCLO Intermediate Risk | 3.65 | (0.93 - 14.35) | 0.102 |
| CCLO High Risk | 9.14 | (2.27 - 36.85) | **0.006** |

[a] HR for PSA-D is reported per 0.10 increase.

CCLO = Cumulative Number of Cancer Locations; CBx = Follow-up Biopsy; Dg = Diagnosis; PSA = Prostate-specific antigen; PSA-D = Prostate-specific antigen density

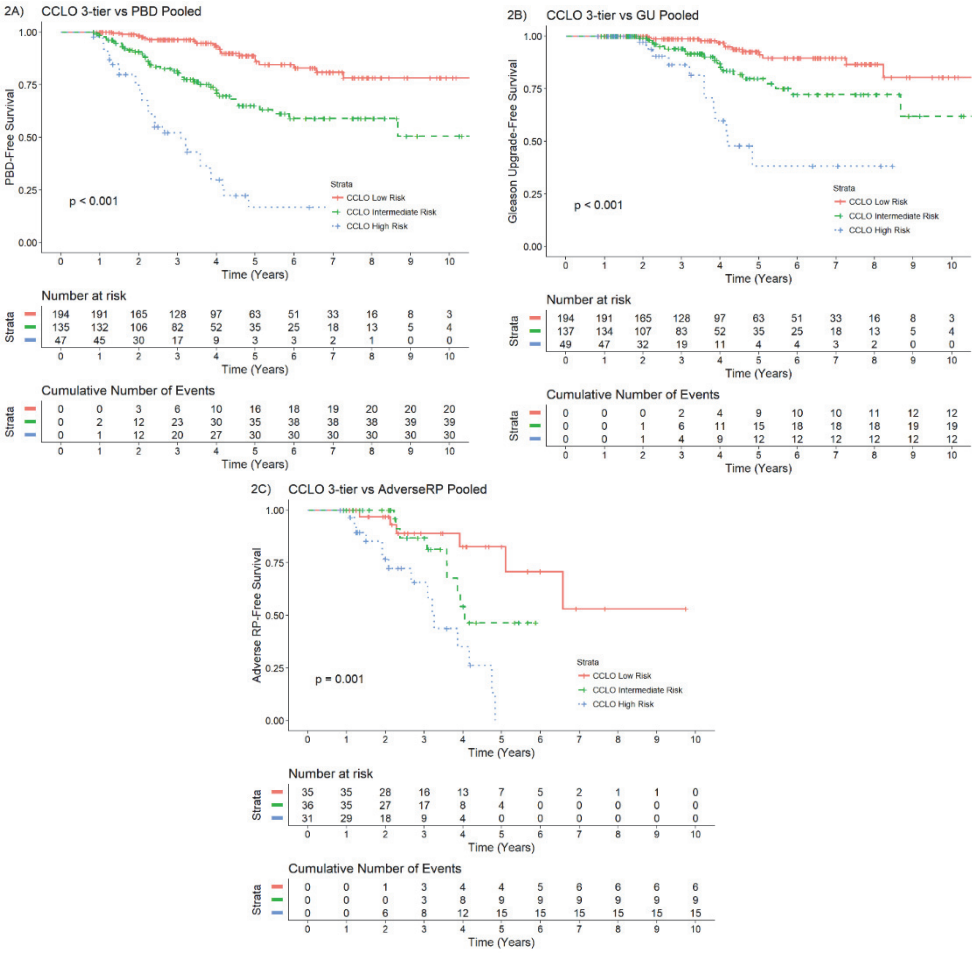


*Figure 24. Kaplan-Meier curves of the 3-tier cumulative number of cancer locations (CCLO 3-tier) risk stratification system for 2A) Protocol-Based Discontinuation (PBD), 2B) Gleason Upgrade-based Discontinuation (GU), and 2C) Adverse findings in RP specimens (Adverse RP). Reprinted with permission from Study I.*

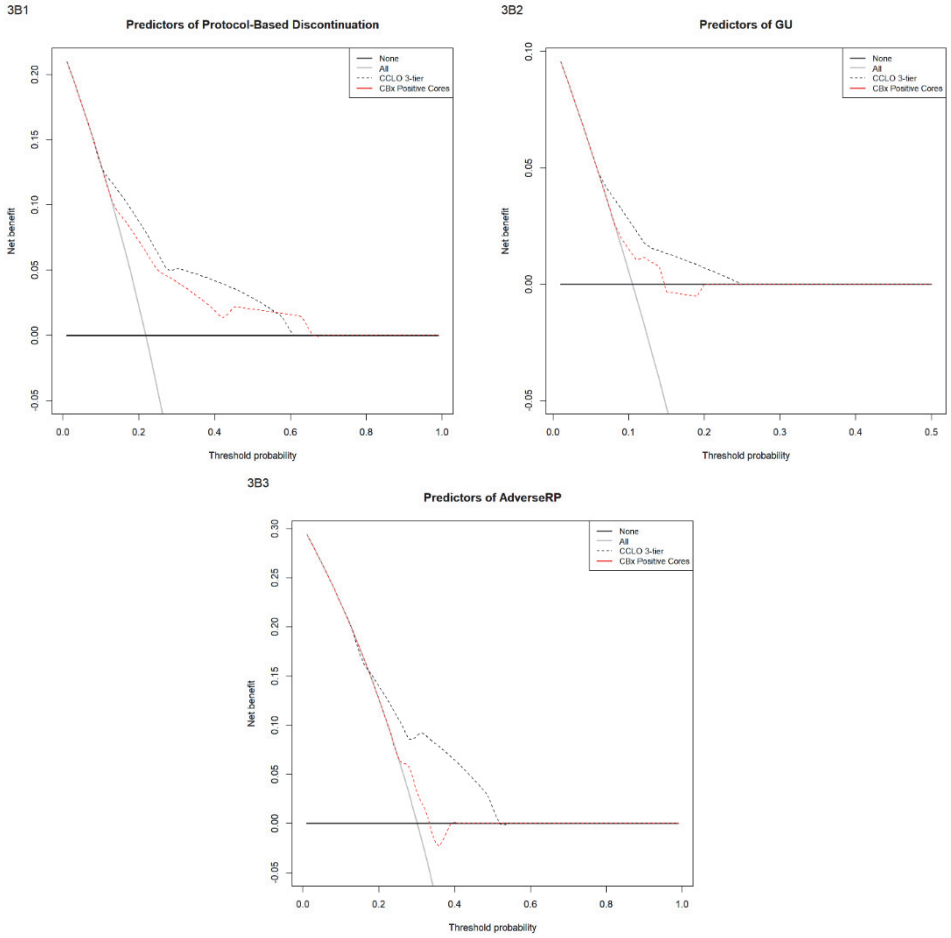


*Figure 25.* Decision curves of the cumulative number of cancer locations 3-tier (CCLO 3-tier) risk stratification and the number of positive core in confirmatory biopsy (CBx Positive Cores) for 3B1) Protocol-Based Discontinuation, 3B2) Gleason Upgrade (GU) and 3B3) Adverse findings in RP specimens. Reprinted with permission from Study I.

## 5.2 Study 2

Of the 203 patients in the study, 190 had representative PTEN stainings, and 190 had representative ERG stainings. Approximately 15% of patients (n = 29) had PTEN loss in diagnostic biopsy, whereas approximately 39% (n = 74) had ERG positivity in diagnostic biopsy (please see Table 1 and Supplementary Table 1 in Original Publication II).

Given our previous findings for ERG and PTEN in radical prostatectomy tissue-microarrays with long term follow-up (Study III), we were interested in the distribution of biomarker status between matched diagnostic biopsy and radical prostatectomy tissue-microarray spots. For Study II, we had matched radical prostatectomy and diagnostic biopsy biomarker status data for 46 patients who underwent radical prostatectomy. In Fisher's chi-squared analyses, diagnostic biopsy and radical prostatectomy ERG status was concordant (P < 0.001), whereas PTEN status was not concordant (P = 0.248) (Table 10).

**Table 10. Concordance of ERG and PTEN IHC status in diagnostic biopsies and RP specimen. Reprinted with permission from Study II.**

| | RP ERG Negative (n = 23) | RP ERG Positive (n = 21) | P-Value |
|---|---|---|---|
| Biopsy ERG Negative | 19 | 6 | <0.001 |
| Biopsy ERG Positive | 4 | 15 | |

| | RP PTEN Negative (n = 5) | RP PTEN Positive (n = 41) | P-Value |
|---|---|---|---|
| Biopsy PTEN Negative | 2 | 7 | 0.248 |
| Biopsy PTEN Positive | 3 | 34 | |

We then wanted to analyze the association between biomarker status and outcome. In Kaplan-Meier analysis of PTEN status and outcome, PTEN Loss was significantly associated with shorter outcome-free survival for all three study outcomes (Figure 26A, C, E, $p < 0.001$). In Kaplan-Meier analyses, ERG status was not associated with outcomes (Figure 26B, D, F). These studies suggest that PTEN status, but not ERG, may be used to stratify patients eligible for AS.

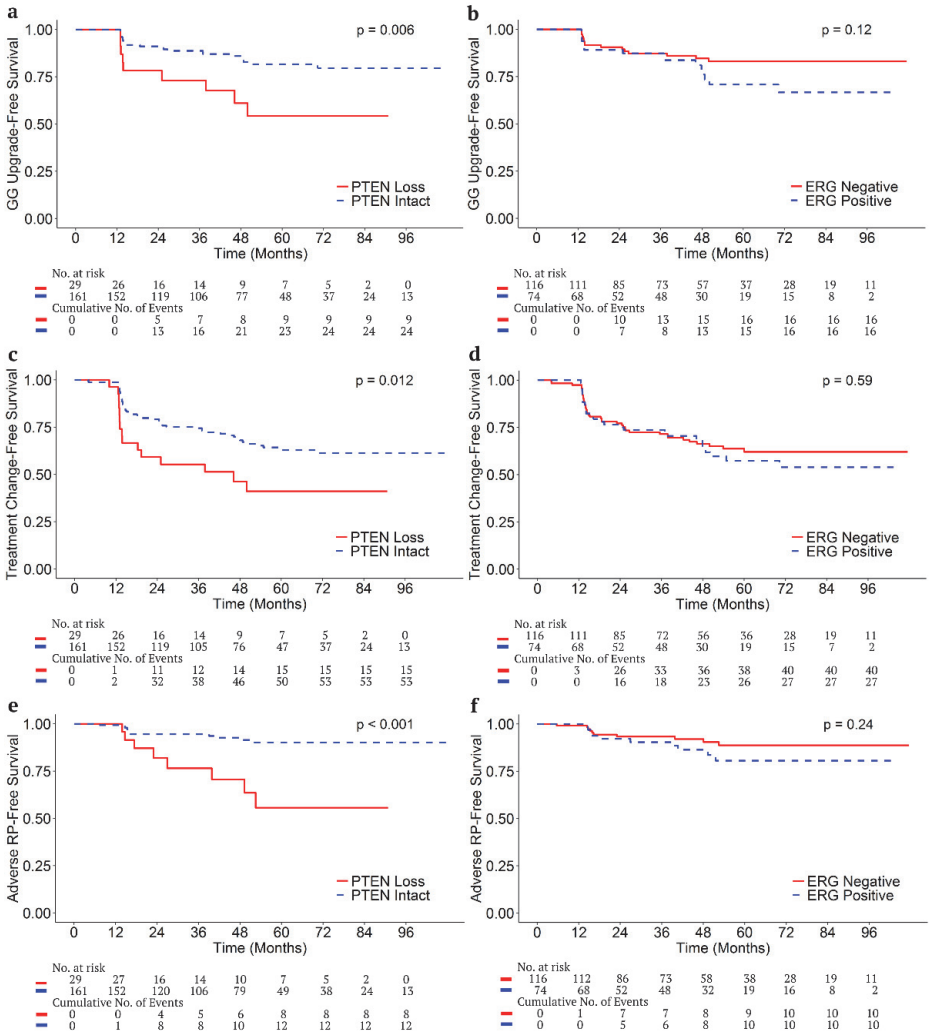


Figure 26. *PTEN loss predicts poorer survival and is associated with worse AS outcomes. Kaplan-Meier curves of biomarker status and outcomes. Grade group upgrade-free survival by A) PTEN status and B) ERG Status. Treatment change-free survival by C) PTEN status and D) ERG Status. Adverse RP-free survival by D) PTEN status and E) ERG Status. Reprinted with permission from Study II.*

Given that patients may discontinue patients AS due to non-protocol based reasons, we conducted Competing Risks Analysis (CRA) to determine whether or not there was a difference in outcomes when stratifying patients by PTEN status. In Gray's CRA, there was a difference in PTEN status was not associated with non protocol-based treatment change, but PTEN status was significantly associated with increased risk protocol-based discontinuation (p = 0.029, Fig. 27).

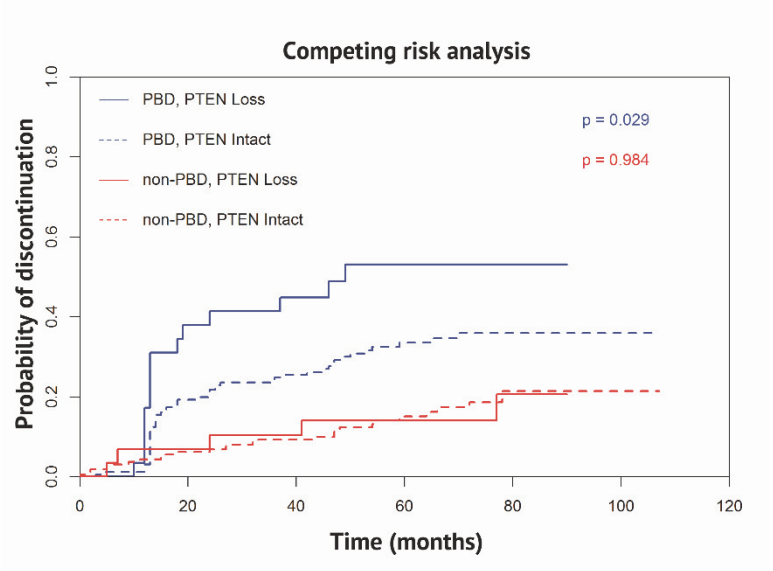


*Figure 27. Gray's competing risk analysis of PTEN status and protocol-based discontinuation (PBD) and non-PBD. Reprinted with permission from Study II.*

In order to determine the independent predictive ability of marker status to predict AS outcomes, we conducted regression modeling of marker status and other standard AS clinicopathological variables. PTEN Loss, but not ERG status, was significantly associated with increased risk for all three study outcomes in both univariate and multivariate Cox Regression analysis (p > 0.015, please see Study II).

## 5.3 Study 3

The study utilized 815 patients samples, of whom 358 were treated with radical prostatectomy in Helsinki, and 457 were treated with radical prostatectomy in Turku. Approximately 17% of patients (n = 135) experienced complete PTEN loss. ERG positivity was found in approximately 50% of all patient's samples (n = 409).

In order to assess the relationship between marker status and relevant clinicopathological variables, we conducted univariate Pearson's chi-squared and Fisher's exact tests. PTEN loss was a significantly associated with all measured variables with the exception of preoperative PSA (Table 11, $p < 0.008$), whereas ERG status was only associated with pre-operative PSA and pTstage (Table 11, $p < 0.008$).

These results suggested that there was a relationship between PTEN status, but not ERG, in predicting outcomes.

In order to analyze the survival profiles of patients by marker status, we conducted Kaplan-Meier analyses. In Kaplan-Meier analysis, patients with any PTEN loss had significantly shorter disease-specific survival than patients with PTEN intact (Not shown, P = 0.011). Furthermore, in Kaplan-Meier analysis, patients with complete PTEN loss had significantly shorter disease-specific survival than patients with partial PTEN loss or PTEN intact (Figure 28, P = 0.017). In contrast, ERG status did not stratify patients by any study outcome (not shown).

**Table 11.** Correlations between ERG and PTEN expressions and clinical variables. Modified and reprinted with permission from Study III.

| | PTEN Status | | | ERG Status | | |
|---|---|---|---|---|---|---|
| | Intact or partial loss (%) | Complete loss (%) | *P value* | Positive (%) | Negative (%) | *P value* |
| All patients (n = 815) | 680 (83.4) | 135 (16.6) | – | 409 (50.2) | 406 (49.8) | – |
| PSA ≤ 10.0 ng/ml | 372 (85.1) | 65 (14.9) | | 237 (54.2) | 200 (45.8) | |
| PSA 10.1-20.0 ng/ml | 157 (84.9) | 28 (15.1) | 0.363[a] | 88 (47.6) | 97 (52.4) | **0.042[a]** |
| PSA > 20.0 ng/ml | 68 (79.1) | 18 (20.9) | | 35 (40.7) | 51 (59.3) | |
| GS ≤ 6 | 243 (93.1) | 18 (6.9) | | 117 (44.8) | 144 (55.2) | |
| GS 7 | 337 (83.4) | 67 (16.6) | **<0.001[a]** | 215 (53.2) | 189 (46.8) | 0.102[a] |
| GS ≥ 8 | 100 (66.7) | 50 (33.3) | | 77 (51.3) | 73 (48.7) | |
| pT2 | 392 (90.1) | 43 (9.9) | | 203 (46.7) | 232 (53.3) | |
| pT3 | 254 (75.6) | 82 (24.4) | **<0.001[a]** | 184 (54.8) | 152 (45.2) | **0.044[b]** |
| pT4 | 1 (33.3) | 2 (66.7) | | 1 (33.3) | 2 (66.7) | |
| LN Positive | 15 (50.0) | 15 (50.0) | **<0.001[b]** | 18 (60.0) | 12 (40.0) | 0.353[b] |
| LN Negative | 659 (84.9) | 117 (15.1) | | 387 (49.9) | 389 (50.1) | |
| Secondary Treatment | 177 (68.1) | 83 (31.9) | **<0.001[b]** | 146 (56.2) | 114 (43.8) | **0.019[b]** |
| No Secondary Treatment | 484 (90.3) | 52 (9.7) | | 252 (47.0) | 284 (53.0) | |
| ACM | 188 (76.7) | 57 (23.3) | **0.001[b]** | 128 (52.2) | 117 (47.8) | 0.446[b] |
| Alive | 492 (86.3) | 78 (13.7) | | 281 (49.3) | 289 (50.7) | |
| PCSM | 36 (69.2) | 16 (30.8) | **0.007[a]** | 26 (50.0) | 26 (50.0) | 1.000[b] |
| Alive or ACM | 644 (84.4) | 119 (15.6) | | 380 (49.8) | 383 (50.2) | |

[a] Pearson Chi-squared test, [b] Fishers-exact test

PSA, prostate-specific antigen; GS, Gleason score; pT, pathological tumor stage; LN, Lymph Node, ACM, Any Cause Mortality, PCSM, Prostate-Cancer Specific Mortality; significant findings in bold

PSA, pTStage, LN, and Secondary Treatment data available for n = 708, n = 774, n = 806, and n = 796 patients respectively

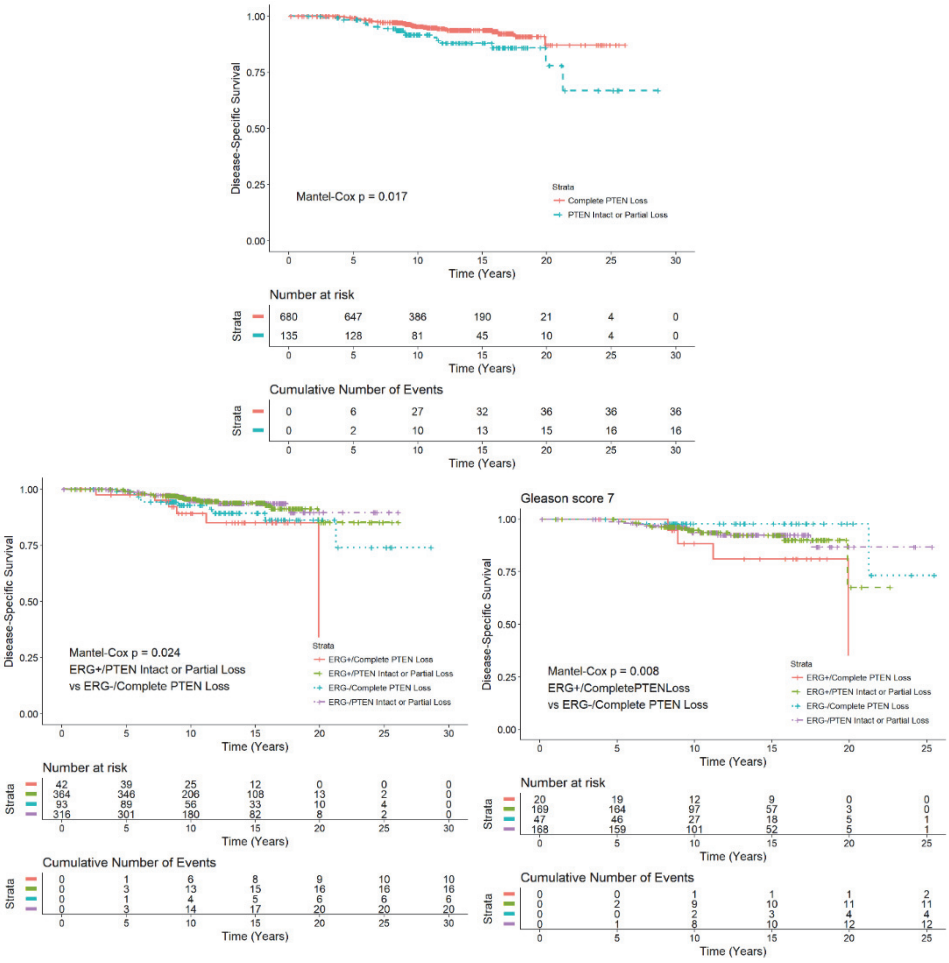


*Figure 28.* Kaplan-Meier curves for A) PTEN status and disease-specific survival (DSS), B) ERG and PTEN status combined with respect to DSS, and C) ERG and PTEN status combined with respect to DSS in Gleason 7 patients. Reprinted in modified style with permission from Study III.

Given that these events can occur in parallel, we analyzed subgroups of combinations of marker status. In Kaplan-Meier analysis of ERG and PTEN status combined, patients who had PC that was ERG negative and had complete PTEN Loss, had significantly shorter secondary treatment-free survival than those with ERG positive and PTEN intact or partial loss PC (Not shown, P = 0.001). Similarly, patients whose PC was ERG negative and had complete PTEN loss, had significantly shorter disease-specific survival than those with ERG positive, PTEN intact/partial loss PC

(P = 0.024). These results suggested that ERG and PTEN status may together predict PC patients with poorer outcomes.

Gleason score 7 PC constitutes the majority of histological findings at the time of radical prostatectomy, and forms an intermediate risk group with a great unmet clinical need with respect to need of prognostic factors to better predict outcomes. With this in mind, we conducted sub-analysis of ERG and PTEN status together in the traditional Gleason scoring system, in GS 7 and GG 3 disease separately. In Kaplan Meier analysis of ERG and PTEN status, patients with ERG negative and complete PTEN loss PC had significantly shorter survival than patients with ERG positive, complete PTEN loss in both GG3 (Not shown, P = 0.023) and GS 7 PC (Figure 28, P = 0.008). These results suggest that ERG and PTEN status may add to the current strongest clinical prognostic factors for predicting poor PC outcomes.

Knowing that AR signaling plays a crucial role in PC development, progression, and treatment, we sought to analyze ERG and PTEN in an AR background. In Kaplan-Meier analysis of patients with ERG negative disease, patients with high AR expression had significantly shorter disease-specific survival than patients with low AR expression (Not shown, P = 0.036). Furthermore, in sub-analysis of patients with ERG negative, AR high disease, patients with complete PTEN loss had significantly shorter disease-specific survival than patients with PTEN intact or partial loss, in both the whole cohort (Not shown, P = 0.007), and in patients with GS7 disease alone (Not shown, P = 0.036).

Lastly, given these findings, we conducted univariate and multivariate Cox regression analysis for clinicopathological variables and marker status with respect to study outcomes. In univariate Cox regression analysis, patients with complete PTEN loss as compared to those with PTEN intact or partial loss at significantly higher risk of prostate cancer-specific mortality and initiation of secondary treatments (Table 12, P < 0.015), however, the finding was only validated with respect to initiation of

secondary treatments in multivariate Cox regression analysis (Table 12, P = 0.003).

**Table 12.** Univariate and Multivariate Cox Regression analyses of clinicopathological variables and outcomes. Reprinted with permission from Study IV

| | Univariate Analyses | | | Multivariate Analyses | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | P-Value | HR | 95% CI | P-Value |
| **A)** Risk of prostate-cancer death (entire cohort, N=815) | | | | | | |
| Age At Dg - < 60.0 | - | - | Reference | - | - | Reference |
| Age At Dg - 60.1-70.0 | 0.98 | (0.55 - 1.76) | 0.951 | 1.09 | (0.28 - 4.29) | 0.904 |
| Age At Dg - >70 | 1.35 | (0.5 - 3.62) | 0.555 | 1.04 | (0.1 - 10.5) | 0.976 |
| Preoperative PSA - 10.0 | - | - | Reference | - | - | Reference |
| Preoperative PSA - 10.0 - 20.0 | 1.86 | (0.79 - 4.4) | 0.156 | 0.73 | (0.12 - 4.47) | 0.73 |
| Preoperative PSA - > 20.0 | 3.78 | (1.57 - 9.07) | 0.003 | 2.05 | (0.55 - 7.67) | 0.288 |
| pTstage 2 | - | - | Reference | - | - | Reference |
| pTstage 3&4 | 14.88 | (5.31 - 41.7) | **<0.001** | 11.84 | (1.33 - 105.33) | 0.027 |
| GS 6 | - | - | Reference | - | - | Reference |
| GS 7 | 18.25 | (2.48 - 134.2) | **0.004** | 7.50E+03 | (0 - 6.5E+109) | 0.943 |
| GS 8-10 | 39.3 | (5.29 - 291.9) | **<0.001** | 3.40E+04 | (0 - 2.9E+110) | 0.933 |
| LN Negative | - | - | Reference | - | - | Reference |
| LN Positive | 7.82 | (3.76 - 16.3) | **<0.001** | 6 | (1.09 - 33.08) | 0.04 |
| PTEN Intact | - | - | Reference | - | - | Reference |
| Complete PTEN Loss | 2.16 | (1.17 - 3.98) | **0.014** | 0.51 | (0.12 - 2.25) | 0.378 |
| Weak AR | - | - | Reference | - | - | Reference |
| Strong AR | 2.38 | (1.01 - 5.6) | **0.048** | 1.41 | (0.35 - 5.72) | 0.632 |
| **B)** Overall Survival (n = 815) | | | | | | |
| Age At Dg - < 60.0 | - | - | Reference | - | - | Reference |
| Age At Dg - 60.1-70.0 | 1.24 | (0.93 - 1.66) | 0.149 | 1.54 | (0.98 - 2.44) | 0.063 |
| Age At Dg - >70 | 3.24 | (2.2 - 4.77) | **<0.001** | 3.25 | (1.79 - 5.9) | <0.001 |
| Preoperative PSA - 10.0 | - | - | Reference | - | - | Reference |
| Preoperative PSA - 10.0 - 20.0 | 0.92 | (0.65 - 1.29) | 0.613 | 0.68 | (0.43 - 1.08) | 0.102 |
| Preoperative PSA - > 20.0 | 1.5 | (1.03 - 2.19) | **0.034** | 0.97 | (0.58 - 1.61) | 0.903 |
| pTstage 2 | - | - | Reference | - | - | Reference |
| pTstage 3&4 | 1.66 | (1.27 - 2.16) | **<0.001** | 1.4 | (0.9 - 2.17) | 0.138 |
| GS 6 | - | - | Reference | - | - | Reference |
| GS 7 | 1.53 | (1.1 - 2.12) | **0.011** | 0.89 | (0.54 - 1.47) | 0.643 |
| GS 8-10 | 2.24 | (1.55 - 3.24) | **<0.001** | 1.42 | (0.74 - 2.72) | 0.298 |
| LN Negative | - | - | Reference | - | - | Reference |
| LN Positive | 3.16 | (1.98 - 5.03) | **<0.001** | 1.62 | (0.49 - 5.37) | 0.431 |
| PTEN Intact | - | - | Reference | - | - | Reference |
| Complete PTEN Loss | 1.32 | (0.97 - 1.8) | 0.081 | 1.02 | (0.63 - 1.65) | 0.94 |
| Weak AR | - | - | Reference | - | - | Reference |
| Strong AR | 1.4 | (1 - 1.97) | 0.053 | 1.1 | (0.72 - 1.67) | 0.672 |
| **C)** Secondary Therapy Free Survival (n = 358, Helsinki Only) | | | | | | |
| Age At Dg - < 60.0 | - | - | Reference | - | - | Reference |
| Age At Dg - 60.1-70.0 | 1.23 | (0.79 - 1.91) | 0.369 | 0.88 | (0.51 - 1.53) | 0.656 |
| Age At Dg - >70 | 1.58 | (0.84 - 2.98) | 0.156 | 0.87 | (0.38 - 2.03) | 0.753 |
| Preoperative PSA - 10.0 | - | - | Reference | - | - | Reference |
| Preoperative PSA - 10.0 - 20.0 | 1.92 | (1.05 - 3.49) | **0.033** | 2.33 | (1.25 - 4.34) | **0.008** |
| Preoperative PSA - > 20.0 | 7.62 | (4.26 - 13.63) | **<0.001** | 5.97 | (3.15 - 11.33) | **<0.001** |
| pTstage 2 | - | - | Reference | - | - | Reference |
| pTstage 3&4 | 4.44 | (2.93 - 6.72) | **<0.001** | 2.25 | (1.32 - 3.82) | **0.003** |
| GS 6 | - | - | Reference | - | - | Reference |
| GS 7 | 5.42 | (2.49 - 11.82) | **<0.001** | 2.22 | (0.89 - 5.53) | **0.087** |
| GS 8-10 | 12.75 | (5.6 - 29.06) | **<0.001** | 6.14 | (2.28 - 16.49) | **<0.001** |
| LN Negative | - | - | Reference | - | - | Reference |
| LN Positive | 4.54 | (1.96 - 10.49) | **<0.001** | 1.05 | (0.31 - 3.48) | 0.942 |
| PTEN Intact | - | - | Reference | - | - | Reference |
| Complete PTEN Loss | 2.78 | (1.85 - 4.19) | **<0.001** | 2.29 | (1.31 - 3.99) | **0.003** |
| Weak AR | - | - | Reference | - | - | Reference |
| Strong AR | 1.29 | (0.84 - 1.96) | 0.245 | 1.12 | (0.65 - 1.92) | **0.691** |

# 5.4 Study 4

A total of 831 patients data were included in the study, with data from 374 patients from Helsinki and 457 from Turku being analyzed.

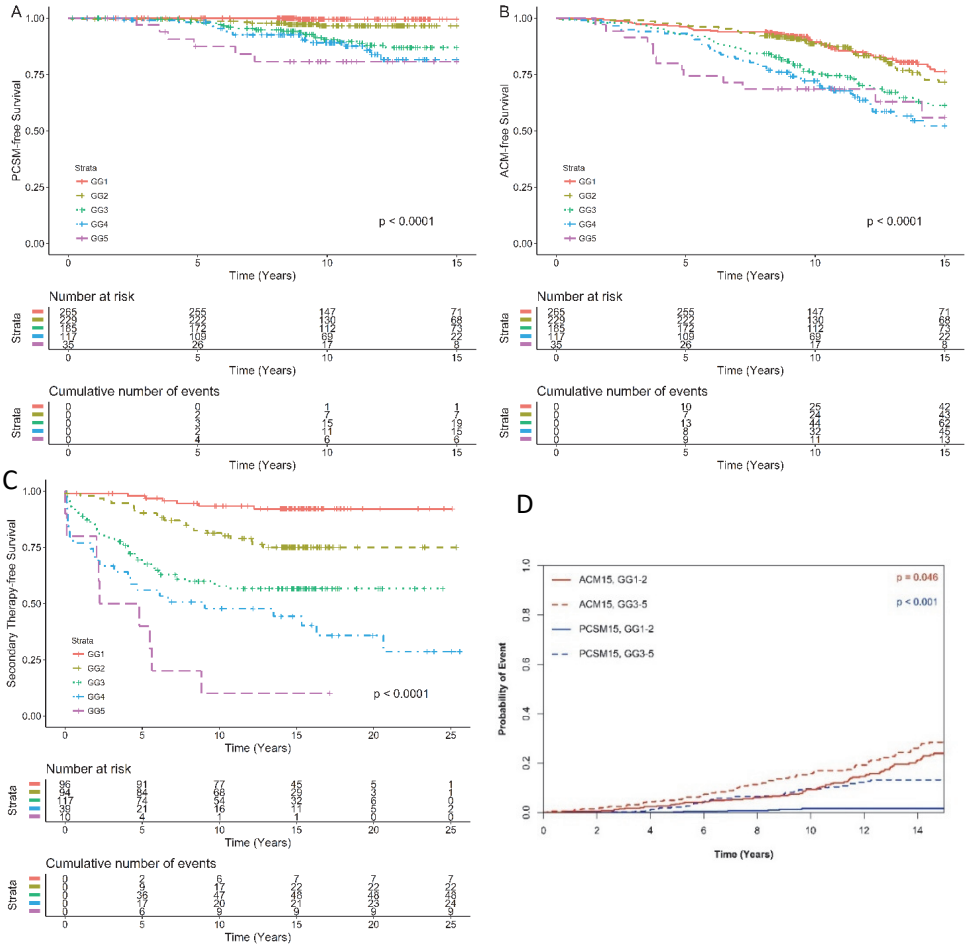


Figure 29. *Survival analyses of grade group and outcomes. A) Kaplan-Meier (KM) curves of prostate cancer mortality-free survival (PCSM) and Grade Group (GG). B) KM curves of any-cause mortality (ACM)-free survival. C) KM Curves of secondary therapy-free survival and GG. D) Gray's competing risks analysis of ACM and PCSM grouped by GG 1-2 and GG 3-5. Reprinted with permission from Study IV.*

In order to analyze the survival profiles of individual GG's in our cohort, we plotted Kaplan-Meier curve. In Kaplan-Meier analysis, higher GG PC significantly predicted shorter prostate cancer-specific mortality, any cause mortality, and initiation of secondary therapies ($P < 0.0001$, Figure 29A-C). Given that any cause mortality is a competing risk for prostate cancer-specific mortality, we conducted Gray's competing risks analysis for any cause mortality and prostate cancer-specific mortality, as stratified by those with GG 1&2 vs GG 3-5 PC. In CRA, there was a difference in survival profiles for patients with GG 3-5 PC as compared to GG 1&2 disease for both any cause mortality and prostate cancer-specific mortality outcomes (Figure 29 D, $P = 0.046$ and $P < 0.001$ respectively). These results confirmed the ability of the new GG system to predict outcomes in our cohort.

In order to determine the independent predictive ability of the new GG system to predict outcomes, we conducted univariate and multivariate Cox regression analysis of GG and other relevant clinicopathological variables. In univariate and multivariate Cox regression analysis, patients with higher GG PC, as compared to those with lower GG PC, were at significantly increased risk of worse study outcomes ($P < 0.05$, Table 13A-C). These results confirmed the independent predictive ability of GG to predict study outcomes in our cohort.

In order to compare the GS (3-tier) and GG 5-tier systems with respect to their ability to predict study outcomes, we conducted ROC-AUC and DCA analyses. In ROC-AUC analyses, GG outperformed GS in predicting prostate cancer-specific mortality (0.764 vs 0.724), any cause mortality (0.631 vs 0.605), and initiation of secondary therapy (0.747 vs 0.720) (not shown). In DCA, GG performed as well as, or better than, GS in all observed threshold probabilities (Figure 30 A-C). These results suggest that GG outperforms GS in predicting PC outcomes in our cohorts.

**Table 13.** Uni- and multivariate Cox regression analysis of clinicopathological variables and outcome. Reprinted with permission from Study IV.

| **Univariate Cox Regression** | | | | **Multivariate Cox Regression** | | | |
|---|---|---|---|---|---|---|---|
| | **HR** | **95% CI** | ***P-value*** | | **HR** | **95% CI** | ***P-value*** |
| **A.** Uni- and multivariate Cox regression analysis of clinicopathological variables and prostate cancer specific mortality within 15 years of follow-up | | | | | | | |
| Age at RP ≤ 60 Years | - | - | Ref | Age at RP ≤ 60 Years | - | - | Ref |
| Age at RP 60.1 - 70 Years | 1.137 | (0.599 - 2.156) | 0.695 | Age at RP 60.1 - 70 Years | 1.908 | (0.799 - 4.553) | 0.146 |
| Age at RP > 70 Years | 1.660 | (0.603 - 4.568) | 0.327 | Age at RP > 70 Years | 0.616 | (0.075 - 5.054) | 0.652 |
| PSA at Diagnosis 0 - 10.0 | - | - | Ref | PSA at Diagnosis 0 - 10.0 | - | - | Ref |
| PSA at Diagnosis 10.1-20.0 | 1.902 | (0.806 - 4.486) | 0.142 | PSA at Diagnosis 10.1-20.0 | 1.514 | (0.623 - 3.678) | 0.360 |
| PSA at Diagnosis > 20 | 2.913 | (1.127 - 7.528) | 0.027* | PSA at Diagnosis > 20 | 1.145 | (0.411 - 3.188) | 0.795 |
| Grade Group 1 | - | - | Ref | Grade Group 1&2 | - | - | Ref |
| Grade Group 2 | 8.015 | (0.986 - 65.14) | 0.052 | Grade Group 3 | 2.699 | (0.861 - 8.464) | 0.089 |
| Grade Group 3 | 27.315 | (3.656 - 204.06) | 0.001* | Grade Group 4 | 6.692 | (2.215 - 20.218) | 0.001* |
| Grade Group 4 | 37.136 | (4.905 - 281.16) | <0.001* | Grade Group 5 | 7.797 | (2.037 - 29.843) | 0.003* |
| Grade Group 5 | 55.799 | (6.717 - 463.52) | <0.001* | pT Stage 2 | - | - | Ref |
| pT Stage 2 | - | - | Ref | pT Stage 3&4 | 6.224 | (1.823 - 21.247) | 0.004* |
| pT Stage 3&4 | 16.544 | (5.088 - 53.8) | <0.001* | LN Negative in first 6 years | - | - | Ref |
| LN Negative | - | - | Ref | LN Positive in first 6 years | 17.481 | (4.883 - 62.582) | <0.001* |
| LN Positive in first 6 years | 16.068 | (6.018 - 42.90) | <0.001* | LN Positive after first 6 years | 1.917 | (0.24 – 15.289) | 0.539 |
| LN Positive after first 6 years | 4.624 | (1.399 – 15.28) | 0.012* | | | | |
| **B.** Uni- and multivariate Cox regression analysis of clinicopathological variables and any cause mortality within 15 years of follow-up | | | | | | | |
| Age at RP ≤ 60 Years | - | - | Ref | Age at RP ≤ 60 Years | - | - | Ref |
| Age at RP 60.1 - 70 Years | 1.217 | (0.882 - 1.681) | 0.232 | Age at RP 60.1 - 70 Years | 1.281 | (0.887 - 1.85) | 0.186 |
| Age at RP > 70 Years | 2.933 | (1.918 - 4.486) | <0.001* | Age at RP > 70 Years | 2.807 | (1.664 - 4.737) | <0.001* |
| PSA at Diagnosis 0 - 10.0 | - | - | Ref | PSA at Diagnosis 0 - 10.0 | - | - | Ref |
| PSA at Diagnosis 10.1-20.0 | 0.967 | (0.667 - 1.402) | 0.858 | PSA at Diagnosis 10.1-20.0 | 0.879 | (0.599 - 1.29) | 0.510 |
| PSA at Diagnosis > 20 | 1.515 | (0.997 - 2.302) | 0.052 | PSA at Diagnosis > 20 | 0.886 | (0.553 - 1.421) | 0.616 |
| Grade Group 1 | - | - | Ref | Grade Group 1 | - | - | Ref |
| Grade Group 2 | 1.181 | (0.772 - 1.807) | 0.444 | Grade Group 2 | 0.928 | (0.569 - 1.513) | 0.763 |
| Grade Group 3 | 2.025 | (1.368 - 2.997) | <0.001* | Grade Group 3 | 1.291 | (0.784 - 2.126) | 0.315 |
| Grade Group 4 | 2.749 | (1.805 - 4.188) | <0.001* | Grade Group 4 | 1.784 | (1.044 - 3.047) | 0.034* |
| Grade Group 5 | 2.861 | (1.536 - 5.331) | <0.001* | Grade Group 5 | 1.823 | (0.864 - 3.845) | 0.115 |
| pT Stage 2 | - | - | Ref | pT Stage 2 | - | - | Ref |
| pT Stage 3&4 | 1.940 | (1.447 - 2.601) | <0.001* | pT Stage 3&4 | 1.520 | (1.056 - 2.188) | 0.024* |
| LN Negative | - | - | Ref | LN Negative | - | - | Ref |
| LN Positive | 3.649 | (2.247 - 5.925) | <0.001* | LN Positive | 3.492 | (1.899 – 6.419) | <0.001* |
| **C.** Uni- and multivariate Cox regression analysis of clinicopathological variables and initiation of secondary therapy | | | | | | | |
| Age at RP ≤ 60 Years | - | - | Ref | Age at RP ≤ 60 Years | - | - | Ref |
| Age at RP 60.1 – 70 Years | 0.832 | (0.481 – 1.439) | 0.510 | Age at RP 60.1 – 70 Years | 1.226 | (0.786 – 1.911) | 0.369 |
| Age at RP > 70 Years | 0.876 | (0.376 – 2.043) | 0.760 | Age at RP > 70 Years | 1.556 | (0.830 – 2.917) | 0.168 |
| PSA at Diagnosis 0 – 10.0 | - | - | Ref | PSA at Diagnosis 0 – 10.0 | - | - | Ref |
| PSA at Diagnosis 10.1-20.0 | 2.152 | (1.162 – 3.983) | 0.015* | PSA at Diagnosis 10.1-20.0 | 1.896 | (1.041 - 3.454) | 0.037* |
| PSA at Diagnosis > 20 | 4.047 | (2.235 - 7.327) | <0.001* | PSA at Diagnosis > 20 | 6.942 | (3.957 – 12.177) | <0.001* |
| Grade Group 1 | - | - | Ref | Grade Group 1 | - | - | Ref |
| Grade Group 2 | 1.676 | (0.626 – 4.487) | 0.304 | Grade Group 2 | 3.376 | (1.442 – 7.903) | 0.005 |
| Grade Group 3 | 2.920 | (1.139 – 7.487) | 0.026* | Grade Group 3 | 7.566 | (3.421 - 16.735) | <0.001* |
| Grade Group 4 | 6.799 | (2.455 - 18.828) | <0.001* | Grade Group 4 | 12.546 | (5.396 - 29.172) | <0.001* |
| Grade Group 5 | 26.208 | (7.785 - 88.23) | <0.001* | Grade Group 5 | 25.591 | (9.478 - 69.097) | <0.001* |
| pT Stage 2 | - | - | Ref | pT Stage 2 | - | - | Ref |
| pT Stage 3&4 | 2.959 | (1.747 - 5.012) | <0.001* | pT Stage 3&4 | 4.586 | (3.039 - 6.919) | <0.001* |
| LN Negative | - | - | Ref | LN Negative | - | - | Ref |
| LN Positive | 1.406 | (0.404 - 4.895) | 0.593 | LN Positive | 4.87 | (2.128 - 11.14) | <0.001* |

**Abbreviations:** RP=radical prostatectomy, PSA=prostate-specific antigen, pT=pathological stage, LN=lymph node.
* Statistically significant *p*-value, 2-tailed

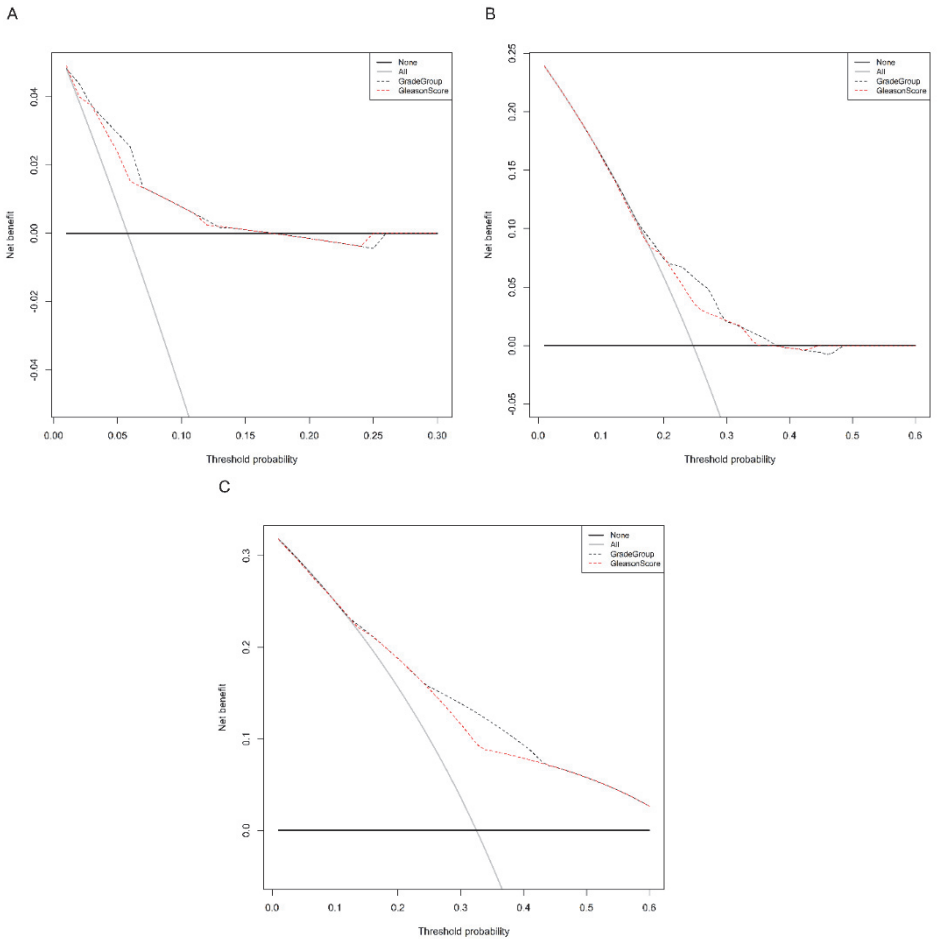


*Figure 30.* Decision curve analyses of Grade Group and Gleason Score and outcomes. Reprinted with permission from Study IV.

# 6. Discussion

## 6.1 Study I

Amin and colleagues wrote in a recent consensus statement, “Although the effect of tumor location at the time of the first diagnosis of cancer and site-specific targeting of positive biopsy locations for determination of subsequent detection/progression of cancer in patients on AS has not been studied, such information may be valuable during further follow-up of those patients (Amin *et al*, 2014).” Taken together, not only does our study suggest that CCLO is a distinct phenomenon from positive cores, but also that CCLO is a powerful predictor of AS outcomes. Given that studies of prognostic factors are often published with minimal increases in predictive power, CCLO demonstrates a stark increase in predictive power over the current clinical standard. To our knowledge, our study is the first to demonstrate that cancer location during the diagnostic phase of active surveillance, is prognostic for PC outcomes.

One prior study by Tseng and colleagues looked at location of PC in diagnostic biopsy and confirmatory biopsy and compared location data with radical prostatectomy findings (Kim *et al*, 2015). The authors utilized an original AS study cohort of 786 men. The authors sub-selected a study cohort of 45 men, who had both diagnostic biopsy, confirmatory biopsy, and radical prostatectomy, whom also had representative PC location data from all time points. Interestingly, the authors found that 71% of patients (n = 32) had discordance between location of PC in diagnostic biopsy and confirmatory biopsy. Furthermore, among these patients, 72% (n = 23) had discordance between diagnostic biopsy PC location and index cancer at radical prostatectomy. In further analyses, authors did not find an association between diagnostic biopsy and confirmatory biopsy location concordance and EPE and radical prostatectomy Gleason score, however, the authors did question whether their studied was sufficiently powered.

Another very recent report by Kearns et al analyzed negative confirmatory biopsy and follow-up biopsies for AS outcomes (Kearns *et al*, 2018). In this study, the authors analyzed 657 AS patients from the multi-center Canary Prostate Active Surveillance Study, and found that having completely negative findings in confirmatory biopsy and first follow-up biopsy was significantly associated with decreased risk of future Gleason upgrading (HR = 0.50, and HR = 0.15 respectively, $P < 0.009$). These

findings are in line with our finding that CCLO-LR are significantly less likely to experience poor AS outcomes. Intriguingly, our results show that even amongst patients with negative confirmatory biopsy, locations distinguish different event-free survival profiles amongst AS patients, warranting further future studies analyzing locations and CCLO even in patients with negative findings.

Our question, whether location of positive cores in the diagnostic phase of AS plays a role in AS outcomes, arose due to contemporary clinical practice, which considers biopsy events as static time points. Our findings are also in line with prior preliminary findings from the nascent PSA era, when quadrant and sextant biopsy procedures were used. The detection rate of PCs rose from 77% to 91% in patients when comparing men who underwent 1 or 2 biopsy procedures respectively (ROEHL *et al*, 2002). However, later studies would demonstrate superior sensitivity of PC detection when utilizing a higher total core number in bx sessions (Gore *et al*, 2001; SINGH *et al*, 2004). Despite these studies, there is ample evidence from studies comparing standard 12-core bx and subsequent radical prostatectomy findings from men who underwent both procedures, that bx can fail to detect relevant foci (Schulte *et al*, 2008; Gallina *et al*, 2012; Iremashvili *et al*, 2012; Washington *et al*, 2012; Lahdensuo *et al*, 2015).

While not studied extensively in the AS context, location and laterality of PC have considered in the context of diagnosis and follow-up of PC. Previous studies have shown that approximately 65% of low-risk PC patients have unilateral bx findings have bilateral PC findings in radical prostatectomy specimens (Scales *et al*, 2007; Isbarn *et al*, 2010). Despite these findings, a majority of patient's bx findings are ipsilateral with extracapsular tumor extension (Sanwick *et al*, 1998), and lymph-node metastases at the time of radical prostatectomy (Weckermann *et al*, 2007; Schiavina *et al*, 2013). Recently, Kim and colleagues looked more specifically at locations, instead of laterality, with regard to positive bx location and location of positive surgical margin at radical prostatectomy, and found that while a majority were concordant, 28% of findings were disconcordant (Kim *et al*, 2015). In studies of AS, laterality was found to be more predictive than maximum core involvement, and subsequently led to the modified Epstein criteria for AS (Kryvenko *et al*, 2014).

Ideally, AS inclusion criteria should be such, that rapidly identifies patients who are at high risk for clinically adverse PC, and direct them to interventions, while at the same time, identifying patients with low-risk

disease as early as possible in diagnostic workup, to prevent comorbidies associated with biopsies (Lahdensuo *et al*, 2016b).

One of the strengths of our study is utilization of a multicenter, international study cohort. Our study featured three different AS cohorts, utilizing two different inclusion criteria. Interestingly, the association between increased CCLO and outcomes held, even considering that one cohort utilized AS inclusion criteria allowing for patients with three positive cores to be included and continue AS.

Our study has a number of limitations, including cohort size. We attempted to stratify patients by CCLO within different groups of patients number of positive confirmatory biopsy. The numbers of patients having two or more cores in confirmatory biopsy was greatly reduced, and likely insufficiently powered to address this question. Additionally, given the increased interest in utilization of the mpMRI, our study did not model CCLO in the context of MRI findings.

Future efforts to study CCLO would include much larger multicenter international validation, perhaps in the GAP3 cohort (Bruinsma *et al*, 2018). Additionally, studies of CCLO should be conducted to assess its predictive role with regard to longer term survival outcomes of PC. Finally, given increased interest in targeted or fusion biopsies, future studies should examine the relationship between CCLO in patients who undergo multiple systematic biopsies and fusion biopsy sessions in an AS context.

## 6.2 Study II

Within the PRIAS internationally across all sites, approximately 52% of men discontinue AS within 5 years, and 73% of men within 10 years (Bokhorst *et al*, 2016). There is an unmet clinical need to better stratify patients into high and low-risk groups earlier during AS. Despite increased interest and evidence for the use of tissue-based markers in predicting PC patient outcomes, few studies have specifically examined tissue-based markers in an AS setting. Our study, to our knowledge, is the first to specifically look at the prognostic value of PTEN protein status in predicting AS outcomes.

A recent study investigated multifocality of localized PC through IHC for ERG and SPINK1 (Fontugne *et al*, 2016). The authors investigated patient's biopsies whose disease was "discontinuously involved" within the biopsy core. The authors found that approximately 1/4$^{th}$ of all such Bx had disconcordant IHC status, suggesting multifocality of PC. While the

authors speculated about the role of multifocality in terms of AS inclusion criteria, the authors did not assess disconcordancy and multifocality with patient outcomes. Future studies should consider assessing the molecular focality (through IHC or genomics) of different CCLO Bx in AS studies, and analyze such marker status with respect to AS outcomes.

Our findings are in agreement with other studies of PTEN status in diagnostic biopsy taken during normal PC diagnosis (not AS). Lotan et al. examined 103 patients who had both GS 6 in diagnostic biopsy and underwent radical prostatectomy, and found that PTEN loss was independently associated radical prostatectomy GS upgrade specimens (Lotan *et al*, 2015). Similarly, Trock et al. utilized a cohort of 150 men in a selected cohort of GS 3+3, 3+4, and 4+3 patients, and analyzed PTEN status only in GS3 regions (Trock *et al*, 2016). Their study found that PTEN loss in the GS3 foci was significantly more likely to occur in the GS3 regions of GS 4+3 and GS 3+4 as compared to GS 3+3 disease. Lastly, a study by Mithal et al. examined PTEN status in diagnostic biopsy from PC patients with long term follow-up (Mithal *et al*, 2014). The authors found that PTEN loss was associated with development of CRPC, PC metastases, and prostate cancer-specific mortality, however, due to the low number of events, it is questionable whether their study was sufficiently powered to address this question. In summary, results from our study concur with prior studies of PTEN status in diagnostic biopsy showing that PTEN loss is associated with higher risk PC and poor outcomes.

Our findings concur with other recent studies investigating both PTEN and ERG IHC in radical prostatectomy specimens. Both the study by Ahearn et al, and our study (Study III) showed that PTEN negative, ERG negative patients fared the worst, in contrast to prior smaller scale studies suggesting that PTEN negative, ERG positive patients fared poorly. These results will be further discussed in section 6.3 (Study III).

Our findings, with regard to ERG status, contrast those from a recent study by Berg et al., who examined the role of ERG status, as determined by IHC, in modeling AS outcomes (Berg *et al*, 2014). The authors studied diagnostic biopsies from a 265 patient cohort of men undergoing AS, with follow up time of 4.1 years. The authors conducted ERG IHC with the exact same antibody as used in our study. While the authors found roughly the same distribution of ERG positivity and negativity (53.6% vs 47.4%), in strong contrast to our findings, the authors found that ERG positivity was independently associated with poor AS outcomes. Considering that

both studies utilized a roughly similar sized number of patients, with similar Northern European genetic and socioeconomic background, the differences in findings between our studies warrant further investigation in the future.

A strength of our study is the utilization of a majority of AS patient tissues taken in our center between 2007-2013, allowing for easier contextualization and clinical translation into the AS setting. An additional strength of our study, is that we utilized two widely validated antibodies specific for PTEN and ERG. Utilization of antibodies in a CLIA approved setting is specific, and cost effective, being orders of magnitude cheaper than other proposed FDA-approved genomic tests for PC.

Our study is limited by the use of a relatively low number of patients in a single institutional setting. Considering our findings and the previous extensive work on PTEN and ERG IHC in many large scale studies, future studies should move from retrospective analysis of real-world data, to prospective studies, ideally utilizing multiple centers and modeling long term outcomes.

## 6.3 Study III

While our study showed that PTEN status was associated with study outcomes (Disease-specific survival, overall survival, and initiation of secondary therapies) in univariate cox regression analysis, the finding was not validated in multivariate analysis. Subsequent to our publication, further work, presented in poster format at the AACR 2017 PC meeting, explored PTEN status in the Turku subcohort analyzed in study III. Additionally, we were able to obtain systematic PSA-data for this cohort, allowing us to model biochemical recurrence. In a multivariable model of CAPRA-S, PTEN status was an independent predictor of outcome (unpublished, not shown). Most commonly utilized multivariable cox regression models, including the approach utilized in our publication, can only model subjects having complete data for all model variables, which led to only approximately 1/3 of all patients being accounted for in analysis. This reduction also reduced the total number of outcome events observed, also likely contributing to our results.

While a majority of studies published to date concur that PTEN loss is associated with poor PC outcomes, the exact prognostic status in an ERG background is controversial. Contrary to our findings, prior studies have found that ERG+/PTEN- disease is associated with poor PC outcomes

(Yoshimoto *et al*, 2008b; Leinonen *et al*, 2013; Hernández-Llodrà *et al*, 2017). Other studies, however, have findings in line with our findings. Reid and colleagues examined PTEN and ERG status by *Fluorescence in situ Hybridization* (FISH) in 308 patients and found that patients with PTEN-/ERG- disease had the worst prognosis (Reid *et al*, 2010). In a similar context, Ahern et al. investigated 1044 radical prostatectomy patients ERG and PTEN status by IHC and analyzed status with respect to 'lethal progression', defined as either metastatic progression or prostate cancer-specific mortality, and found that patients with PTEN-/ERG- disease were at significantly higher risk of poor outcome (Ahearn *et al*, 2015). Further studies are needed to fully elucidate the prognostic role of combined ERG/PTEN status.

One possible explanation for differing outcomes may be ethnic background. A study by Mao et al. found that the rate of 21q22.2-22.3 deletion (responsible for approximately 50% of TMPRSS2:ERG fusions in Western populations) was detected at a significantly reduced rate in Asian populations (Mao *et al*, 2010). Similarly, a recent study by Tosoian et al. found that alterations of PTEN and ERG occurred at a reduced rate in African-American males as compared to European-Americans (Tosoian *et al*, 2017). While the Finnish population is primarily ethnically homogenous (Kerminen *et al*, 2017), unlike other many other countries in the world, Finland has systematic registries documenting residency and migration patterns (Basic information). Such data could, and should be used in the future to examine biomarker differences, including PTEN and ERG between native born, ethnic Finns and immigrants and examine whether these differences play a role in Finnish PC patient outcomes.

Strengths of our study include the use of previously validated antibodies that have been widely used in other studies of PTEN and ERG protein expression status. Furthermore, our study utilized robust Finnish Cancer Registry outcome data allowing for long-term follow-up to be modelled. Furthermore, our study utilized multiple center's samples and study data.

Our study is limited by its retrospective nature. Prognostic studies analyzing PC are difficult due to the long follow-up time needed to observe relevant outcomes. While our study addressed this issue by utilization of registry data, our patients came from two cohorts, one treated between 1983 – 1998, and the other from 2000-2005. In the first study cohort, 1/3 of the patients underwent primary treatment prior to the routine utilization of PSA, which as described previously, transformed the diagnostic workup of PC. Furthermore, the changes in clinical care since time of treatment

raise the question of modeled outcomes in a modern context. These criticisms, however, are inherent in studying any prognostic factors of PC. If one wishes to model shorter term outcomes in a modern study cohort, investigators run into the issue of whether or not clinically relevant outcomes are truly modeled in their study.

Our results, considered in the context of other recently published studies add to a conclusive body of evidence identifying PTEN status as a significant independent predictor of PC outcomes. Future efforts should focus on incorporating PTEN status into routine clinical practice or pursuing study designs that would consider marker status in diagnostics. Prospective randomized trials utilizing diagnostic models of standard clinicopathological variables with and without PTEN status should be strongly considered. Furthermore, efforts to understand, mitigate and/or reverse the biological mechanisms underpinning these changes should be further explored for clinical utility.

## 6.4 Study IV

To our knowledge, our study is the first to meaningfully demonstrate that indeed GG is more predictive for mortality outcomes than GS.

Finnish cancer registry PC mortality outcomes data are of exceptionally high quality, with a previous study of the Finnish subset of the ERSPC PSA-screening trial showing 96.1% sensitivity, and 98.9% specificity for correctly encoded outcome (Mäkinen *et al*, 2008). In contrast, a similar historical review of mortality-specific outcomes in the United States suggested a potential error rate of 10-20% (Albertsen *et al*, 2005). Even with accurate outcomes data, however, utilizing of PC-specific mortality, while sensitive, is not specific, as half of men with clinically relevant PC die due to other causes. Given that less than 15% of men die due to disease, long term follow-up is needed to observe survival outcomes. This has led to the use of surrogate outcomes, such as biochemical recurrence (D'Amico *et al*, 2003) for modeling outcomes. These surrogate outcomes, however, often do not reflect true outcomes, as 15-30% of all primarily treated patients experience biochemical recurrence (Han *et al*, 2001; Cooperberg *et al*, 2011b).

Previous studies comparing GG and GS had mainly focused on modeling biochemical recurrence outcomes from patients treated with primary radical prostatectomy. In these studies, GG outperformed GS with respect

to predicting biochemical recurrence (Loeb *et al*, 2016; Spratt *et al*, 2016; Mathieu *et al*, 2017; Yeong *et al*, 2017).

In contrast to the aforementioned studies, two other recent studies focused on analyzing the discriminatory power of GG as compared to GG with harder clinical outcomes. Dell'Oglio and colleagues conducted a large retrospective study of patients treated between 2005 and 2014 in two separate centers in North America and Italy (Dell'Oglio *et al*, 2017). The authors analyzed radical prostatectomy GG vs GS with respect to clinical recurrence, defined as "positive imaging during follow-up after the onset of biochemical recurrence" and found that GG did not out perform GS. While having an impressive study size, the authors did not specify whether re-review of slides to the updated GG standard was performed, or whether their study relied on refactoring the contemporary GS assigned during routine clinical work. In a study by Grogan and colleagues, they performed central re-review of a cohort of 635 radical prostatectomy patients treated between 1991 and 1999 in a single center (Grogan *et al*, 2017). In their study, they compared GG vs GS with respect to biochemical recurrence, clinical recurrence, and prostate cancer-specific mortality. In line with our findings, the authors found increases Harrell's c-indices (effectively similar to ROC-AUC employed in our study), in GG vs GS for all study outcomes. In contrast to our study, however, the authors did report that GG was an independent predictor of prostate cancer-specific mortality in multivariable analysis.

Strengths of our study include central-review of slides, fairly large number of patients, multicenter cohorts, long follow-up time, and highly accurate Finnish Cancer Registry outcome data. Our study is limited by the fact, that given that many study patients were treated during 1980's-early 2000's, the diagnostics and follow up treatments have significantly changed since this time.

# 6.5 Synthesis

Taken together, the Studies I & II present two novel predictors for consideration in future AS criteria. These studies have been conducted at the time of an interesting crossroads with regards to prostate cancer active surveillance. Clinical practice in the diagnostic workup of PC is changing with the introduction of new imaging modalities that were not mature at the time of first conception of AS in the mid 2000's. Underlying these changes is the key concept of how to best identify patients with clinically significant prostate cancer as early as possible, while minimizing the ratio

of identification of patients with clinically insignificant prostate cancer. Undoubtedly, the exact form of AS, with regard to the modality and timing of imaging and biopsying is being investigated and will change in the future. Despite the lack of knowledge of the final form of these changes, all forms of the diagnostic work-up of PC will continue to rely on prostate biopsy, necessitating clinicopathological evaluation of PC tissue.

In this regard, many future studies examining the association CCLO and biomarkers with patient outcomes are possible. With regards to cumulative cancer locations (Study I), some clinics around the world will not have access to magnetic resonance imaging or fusion biopsy devices in the near future, implying that patients may undergo AS series utilizing TRUS-guided biopsies only. CCLO could potentially be utilized to aid in the follow-up of these patients. Larger scale studies, validating the difference between positive cores and CCLO, as well as analyzing the association with harder outcomes is also necessary.

In contrast, given the rapidly adoption of magnetic resonance imaging and fusion biopsy in academic medical centers in developed countries, future AS series in these centers will likely utilize more targeted biopsies (and subsequently less bi-sextant TRUS biopsies). The current reporting and follow-up of AS in these patients, however, also does not account for the location of PC in modeling risk of outcomes. Similarly, given that current AS models utilize a ratio of positive to negative cores, targeted biopsies will increase the number of positive cores. Given the findings of our study, how should PC location be incorporated into the clinical decision making for these patients? It is possible that patients may undergo systematic biopsy, prior to, in parallel, or after targeted biopsy. Subsequently, there may be patients who are disconcordant in location of PC detected in targeted biopsy and systematic biopsy. Will the outcomes of these patients differ than those whose PC is concordant? More studies to address these questions are warranted.

Similar questions arise with consideration of the findings of PTEN IHC (Study II). While validation is necessary, current evidence strongly indicates that PTEN loss, at any stage of PC progression, is associated with poor PC outcomes. Given that PTEN immunohistochemistry status in systematic biopsies was not concordant with PTEN immunohistochemistry status in radical prostatectomy, in the setting of fusion biopsy, will PTEN immunohistochemistry findings be concordant with radical prostatectomy? Are biomarker status and location concordant between patients with differing levels of CCLO? More importantly, would

potential differences better predict outcomes? Prospective studies utilizing a model of AS incorporating both biomarker status and CCLO should be performed.

Studies III & IV present evidence for the use of novel clinical and biomarker data in prognostication of longer term PC outcomes in patients primarily treated for PC. Will these studies, however, change current clinical practice? As compared to studies I & II, the answer is more clear. Considering the results from Study III, in addition to our findings, there is independent validation that PTEN status predicts long term outcomes of PC. How should a treating physician consider these findings, and would it change their treatment? Given the retrospective nature of our study, despite clear indication that biomarker status predicts outcomes, at this time it is impossible to give recommendations for specific intervention based on these findings. Prospective randomized trial(s) to show that intervention immediately after primary therapy, based on marker status, should be considered. A less mature, but a potentially more revolutionary intervention, would be the consideration of targeted therapies to prevent, or reverse molecular changes induced by PTEN loss and ERG positivity. In contrast to the current clinical implications of Study III, the considerations from Study IV are much more clear; our study provides evidence that GG outperforms GS in predicting outcomes of PC, and provides an evidence-based rationale to utilize GG instead of GS. Future studies utilizing a multi-center approach and comparing GG and GS in the same patients should be performed to confirm our findings.

# 7. Conclusions

Brief summary conclusions of the four publications are outlined as follows:

**Study I:** The cumulative number of cancer locations between diagnostic biopsy and confirmatory biopsy is distinct from the number of positive cores. Increased CCLO was associated with poor outcomes in two different AS criteria at three independent centers. In a multivariable model containing other standard AS inclusion criteria, increased CCLO was an independent predictor of poor AS outcomes. In discriminatory analysis, CCLO better predicted AS outcomes than the number of positive confirmatory biopsy (the current clinical standard). Taken together, CCLO is a simple metric to better predict AS outcome and aid in patients selection during the initial diagnostic period of one year after primary diagnosis.

**Study II:** PTEN loss, but not ERG status, in diagnostic biopsy of AS patients, was associated with poor AS outcomes. PTEN loss was an independent predictor of poor AS outcomes in a multivariable model containing other standard AS variables.

**Study III:** In a multi-center study, PTEN loss was associated with decreased secondary therapy-free survival and disease-specific survival. PTEN negative, ERG negative, AR high PC's had the poorest outcome.

**Study IV:** In a multi-center study with long term follow up, Grade group was independently associated with shorter secondary therapy-free survival and mortality. In discriminatory analyses, GG outperformed GS in predicting mortality outcomes.

**Synthesis and Clinical Recommendations:** Grade group outperforms Gleason score in predicting prostate cancer patient outcomes, and should be adopted into routine uropathological assessment of prostate cancer. In considering candidates for active surveillance, PTEN stainings should be performed on diagnostic biopsies, and if PTEN loss is found, treatment change to radical intervention should be considered. If a patient continues on AS to confirmatory systematic biopsy, the number of cancer locations between diagnostic and confirmatory biopsies should be assessed, and if patients are determined to be in the high risk group of CCLO ≥ 3, treatment change should be considered. Finally, if PTEN loss is found in patient's radical prostatectomy specimens, these patients should be followed more intensively post-operatively. However, further clinical validation is needed, preferably in prospective randomized trials.

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# 10. Original Publications